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Ion-pairing separation of bioactive peptides using an aqueous/octan-1-ol micro-extraction system from bovine haemoglobin complex hydrolysates

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ABSTRACT

The ion-pair concept was applied on complex haemoglobin hydrolysates to extract two opioid peptides, LVV-haemorphin-7 and VV-haemorphin-7, in an aqueous/octan-1-ol micro-extraction system in the presence of alkyl-sulfonic acid as a surfactant agent and in relation to the haemorphin physico-chemical properties (charge, hydrophobicity). The effect of combined alkyl chain length/aqueous phase pH and the haem behaviour during the extraction, on the haemorphin recovery yield and enrichment has been determined. It has proved that transport over the organic phase is mediated by the alkyl-sulfonic acids, whatever be the aqueous phase pH. However, increasing both the alkyl chain length and the pH in the aqueous phase shows an haemorphin enrichment ratio increase but a recovery decrease of the extracted opioid peptides in the organic phase. Therefore, the best conditions to extract LVVh-7 and VVh-7 are the use of the octane-sulfonic acid at aqueous phase pH of 5 or 7 and the octane or the heptane-sulfonic acid with an aqueous phase pH of 5 or 7 respectively. In these conditions, a partition coefficient of 1.64 and 1.60 respectively for LVVh-7 and VVh-7 are obtained and represent about 40 times that acquired without agent.

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1. Introduction

Enzymatic hydrolysis of proteins is widely used in the food industry to improve functional properties such as solubility, emulsification, gelation and taste, or to prepare extensively hydrolysed proteins for hypoallergenic infant diets and nutritional therapy [1,2]. More recently the pharmaceutical and food industries have shown increasing interest in peptides derived from protein hydrolysates as components of functional foods [3,4]. Much research has focused on the isolation of peptides derived from various protein sources (milk, soya, fish, ...). Numerous biologically active peptides (antihypertensive, opioid, antibacterial, ...) have already been obtained by hydrolysis of casein, soya proteins, gluten, tuna myoglobin and others [3–11].

The preparation of such peptides or peptide-containing fractions generally required laborious chromatographic steps including pre-fractionation methods as gel filtration and reverse phase liquid chromatography [12,13]. Therefore, replacement of even one column chromatographic operation by a simple-yet effective-separation step in an overall purification scheme could significantly reduce the expense of producing peptides of interest. Moreover, development of processes to prepare pure active peptides or enriched active peptide fractions is very difficult because these peptides are in a very complex mixture, i.e. an hydrolysate containing more than hundred peptides whose sequence is different.

LVV-haemorphin-7 (LVVh-7) and VV-haemorphin-7 (VVh-7) were isolated and characterized for the first time in our laboratory [14]. These peptides correspond respectively to residues 31–40 (Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe) and 32–40 (Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe) of bovine haemoglobin β-chain. The hydrophobic properties of these two peptides, via their sequences, have allowed their solubilisation in aliphatic alcohol mixture butan-2-ol and octan-1-ol. Mixture design has then been used to improve the extraction of LVVh-7 and VVh-7 in the course of the haemoglobin hydrolysis by pepsin at pH 3 by a mixture of butan-2-ol and octan-1-ol. A biphasic system composed of 45% water, 45% butan-2-ol and 10% octan-1-ol was proposed [15]. However, even if a good selectivity was obtained, with only four extracted peptides including the two haemorphins, low extraction recovery was observed due to the low haemorphin partition coefficient (about 0.32).

Hydrophobic ion pair has been often used for non-aqueous enzyme reactions [14–16] or enhanced protein stability in organic solvent [17,18]. Major advantage of hydrophobic ion-pairing is to

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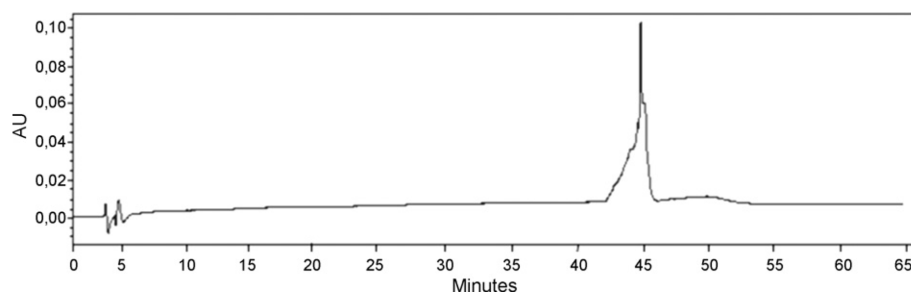

Fig. 1. RP-HPLC profile of octan-1-ol. 50 μL of organic solvent were fractionated on C4-column.

increase the lipophilicity of hydrophilic molecules [17,19]. Another strategy has also been developed for the separation of peptides or proteins by ionic-pairing with amphipathic molecules [20–23]. Hydrophobically modified peptides or proteins allowed the increase of their extraction in organic solvent. For mixtures containing more than hundred different peptides, this strategy appears a good means to separate peptides by modifying their hydrophobicity thanks to ion pair agent–peptide complex formation and in using liquid/liquid medium to selectively extract some bioactive peptides. To allow this study, two opioid peptides, LVV-haemorphin-7 and VV-haemorphin-7, have been chosen because previous studies have shown that these peptides can be selectively extracted in a biphasic system containing butan-2-ol–octan-1-ol but a very low partition coefficient is obtained [24,25]. Therefore, ion-pairing extraction has been applied with alkyl-sulfonic acids, chosen as acidic amphipathic surface-active agents, to increase the lipophilicity of the active peptides via hydrophobic ion-pairing. Action of the alkyl-sulfonic acid counter anions as lipophilicity modulator at different pH and with different alkyl chain lengths, and a partition coefficient comparison with and without alkyl-sulfonic acid have been discussed in previous studies [24,25] and have demonstrated the potential of this system for selective extraction of peptides.

2. Experimental procedure

2.1. Materials

All common chemicals and solvents were of analytical grade from commercial sources. Bovine haemoglobin was spray-dried beef haemoglobin powder from Vapran SA (France). Pig pepsin [E.C. 232-629-3, 3260 UAE/mg solid] and alkyl-sulfonic acid sodium salts were purchased from Sigma. Acetonitrile was of HPLC grade. All aqueous HPLC eluent were degassed with helium.

2.2. Hydrolysate preparation

Bovine haemoglobin hydrolysis by pepsin was performed at 23 °C at pH 3 with pH-stat. Haemoglobin solution (2%, w/v) was incubated with pig pepsin (E/S = 2.5%, w/w) for 2.5 min and 10 h. Reaction was stopped by inactivating pepsin by increasing the pH to 10 with 5 M NaOH.

2.3. Determination of the haemoglobin hydrolysis degree

The degree of hydrolysis was defined as the ratio of the number of peptide bonds cleaved to the total number of peptide bonds in haemoglobin determined by the trinitrobenzene sulphonate method [30]. 2.5 min and 10 h of hydrolysis by pepsin corresponded to DH 3% and 15% respectively [26].

2.4. Haemorphin ion-pair micro-extraction

Ion-pair micro-extraction was carried out in a liquid–liquid biphasic system, with aqueous and organic phases at equal volumes (400 μL). The aqueous phase contained the DH 3% or 15% hydrolysate (1%, w/v) and alkyl-sulfonic acid with a 6, 7 or 8 carbon chain length (50 mM), in the presence of 50 mM phosphate solution. Alkyl-sulfonic acid with 9 carbons or more were not

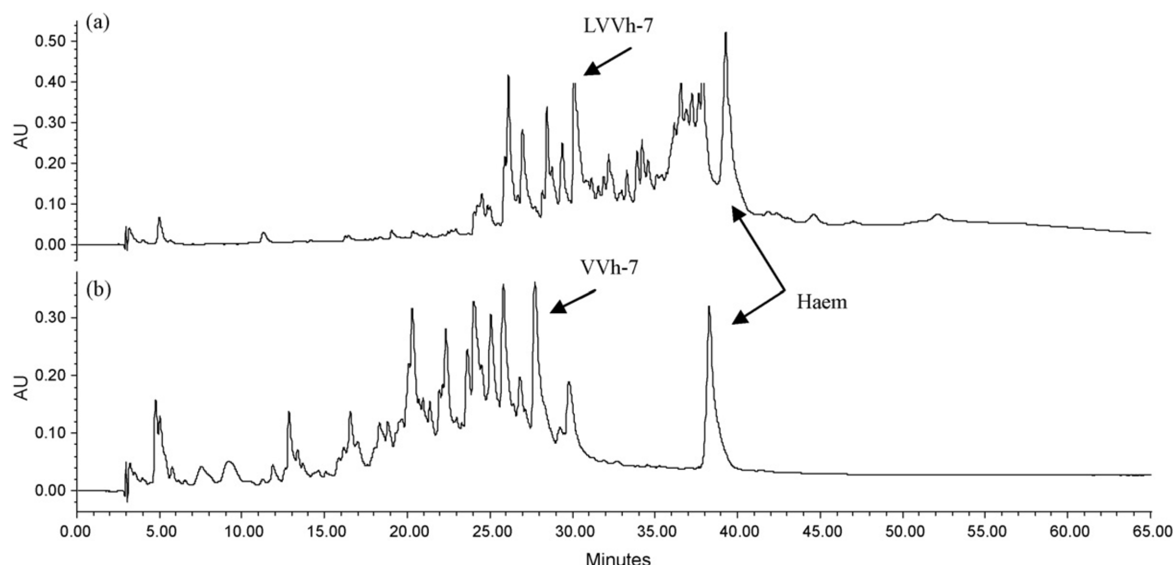

Fig. 2. RP-HPLC profiles of peptidic hydrolysates at (a) 3% and (b) 15% of hydrolysis degree. 40 μL of solution (1%, w/v) were fractionated on C4-column.

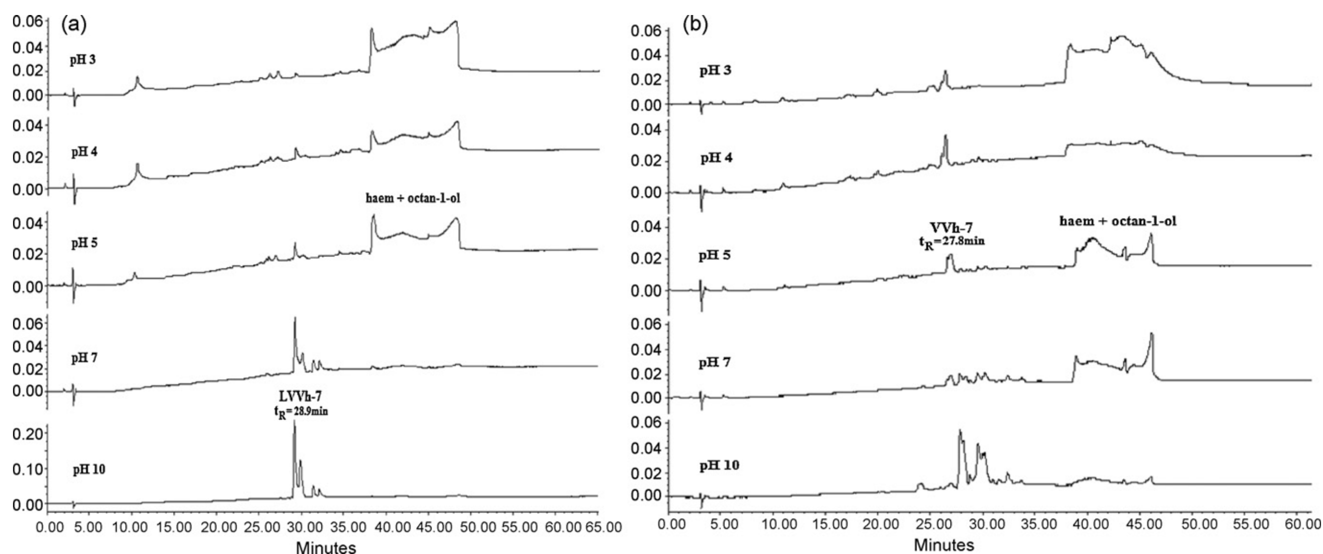

Fig. 3. RP-HPLC profiles of the organic phases after ion-pair micro-extraction without alkyl-sulfonic acids and at pH 3, 4, 5, 7 and 10 with the (a) DH 3% hydrolysate and (b) DH 15% hydrolysate. 100 μL of organic phase were fractionated on C4-column.

applicable because of their bad solubility in aqueous solution. pH from 3 to 10 was adjusted with HCl or NaOH. Below pH 3, haem present in the hydrolysate immediately precipitate causing a loss of peptides in solution. The organic phase was composed of octan-1-ol. The extraction was performed at room temperature for 12 h (preliminary studies have shown that there was no difference in extraction recovery between 6 and 24 h) on a Rotator SB3 (Stuart®) at 17 rpm. After extraction, the sample was centrifuged at 5000 rpm for 10 min, and the organic phase was collected and directly analysed by RP-HPLC. For all experiments, LVVh-7 and VVh-7 extraction yields and enrichments were determined for DH 3% and 15% respectively.

2.5. Analytical methods

2.5.1. RP-HPLC analysis

HPLC apparatus consisted of a Waters 600^E automated gradient controller pump module, a Waters Wisp 17 automatic sampling device and a Waters 996 photodiode array detector. Spectral and chromatographic data were stored on a NEC Image 466 computer. Millennium software was used to plot, acquire and analyse chromatographic data. All the chromatographic process was performed with a Vydac C4-column (250 mm × 4.6 mm internal diameter). The mobile phase was water/trifluoroacetic acid (1000:1, v/v) as eluent A and acetonitrile/water/trifluoroacetic acid (600:400:1, by volume) as eluent B. The flow rate was 1 mL/min. The gradient applied was 0–67% (v/v) B over 30 min then 67–87% (v/v) B over 35 min. On-line instantaneous UV absorbance scans were performed between 200 and 395 nm with photodiode array detector at a rate of one spectrum every second. The resolution was 1.2 nm.

2.5.2. Haemorphin and haem quantification

Identification and quantification of expected haemorphins and haem in the organic phases were carried out by UV-spectral comparison and standard curves with the Millennium software, as described previously [24]. Distortion of the octan-1-ol peak shape (retention time about 45 min) did not influence haemorphin detec-

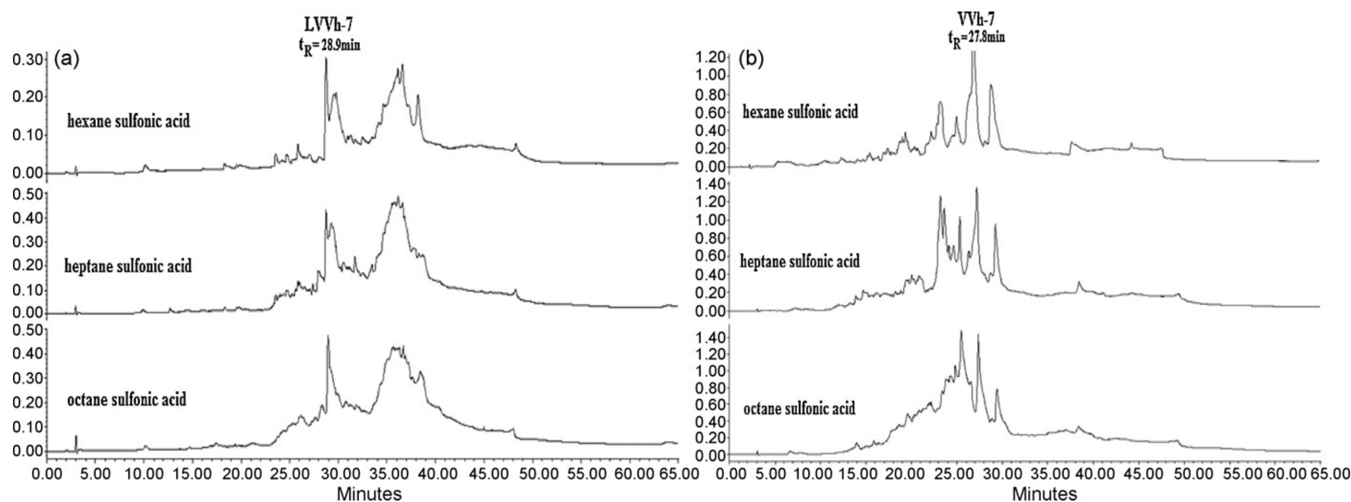

Fig. 4. RP-HPLC profiles of the organic phases after ion-pair micro-extraction in the presence of hexane, heptane or octane-sulfonic acid and at pH 3 with the (a) DH 3% hydrolysate and (b) DH 15% hydrolysate. 100 μL of organic phase were fractionated on C4-column.

tion and quantification by the Millennium software. Fig. 1 shows a peak with a retention time of about 45 min which corresponds to the octan-1-ol solvent.

2.6. Response measurement

The enrichment ratio (ER) and recovery (R) of the haemorphins were the response variables used to analyse the experiments.

The haemorphin ER was calculated using Eq. (1):

$$\mathrm{ER} = \frac{[(\%_{\mathrm{h}})_{\mathrm{final}}]_{\mathrm{O}}}{[(\%_{\mathrm{h}})_{\mathrm{initial}}]_{\mathrm{A}}} \quad (1)$$

- $[(\%_{\mathrm{h}})_{\mathrm{final}}]_{\mathrm{O}}$ (percentage of haemorphin in final organic phase) is the ratio of the haemorphin-containing fraction area in the organic phase after ion-pair extraction on the extracted peptide total area in the organic phase after the extraction.
- $[(\%_{\mathrm{h}})_{\mathrm{initial}}]_{\mathrm{A}}$ (percentage of haemorphin in initial aqueous phase) is the ratio of the haemorphin-containing fraction area in the aqueous phase before the extraction on the peptide total area in the aqueous phase before ion-pair extraction.

Therefore, the ER factor is an evaluation of the purification degree of extracted haemorphin in the organic phase and it allows to determine the selectivity of the extraction system.

The haemorphin R (%) was evaluated with Eq. (2):

$$R = \frac{[(\mathrm{C}_{\mathrm{h}})_{\mathrm{final}}]_{\mathrm{O}}}{[(\mathrm{C}_{\mathrm{h}})_{\mathrm{initial}}]_{\mathrm{A}}} \times \left(\frac{V_{\mathrm{O}}}{V_{\mathrm{A}}}\right) \times 100 \quad (2)$$

where $[(\mathrm{C}_{\mathrm{h}})_{\mathrm{final}}]_{\mathrm{O}}$ is the haemorphin concentration in the organic phase after ion-pair extraction, $[(\mathrm{C}_{\mathrm{h}})_{\mathrm{initial}}]_{\mathrm{A}}$ is the haemorphin concentration in the aqueous phase before the extraction and V_{O} and V_{A} are the volumes of the organic and aqueous phase respectively (in our experiments the volumes are equal).

It was also possible to obtain a high haemorphin R but a low ER because many other peptides are extracted in the organic phase with the haemorphins.

All areas were obtained thanks to the Millennium software and all calculations were carried out using three parallel determinations.

3. Results and discussion

3.1. Recovery in the absence of alkyl-sulfonic acids

The experiment was carried out to check if some peptides in the absence of a carrier ion can pass the liquid–liquid interface. The tested hydrolysates were a DH 3% hydrolysate for LVVh-7 and a DH 15% for VVh-7 because these opioid peptides are predominantly present in these peptidic mixtures [9]. Extraction was performed with octan-1-ol as the organic phase because it is a water immiscible solvent and it is the reference for the determination of the partition coefficient of biomolecules [27]. Moreover, alcohol solvents have shown a better extraction towards the two haemorphins than solvents less polar [28]. The aqueous pH varied from 3 to 10 and the ionic strength was maintained thanks to 50 mM of phosphate salt during the extraction. Fig. 2 shows chromatographic profiles of the DH 3% and 15% hydrolysates respectively. Haem molecule was detected with a retention time of 38.4 min. LVVh-7 was detected in DH 3% hydrolysate with a retention time of 28.9 min and VVh-7 in DH 15% hydrolysate with a retention time of 27.8 min. These peptides contained an arginine basic residue in their sequence and had a hydrophobicity of about 1700 cal/mol, as the Bigelow scale [27], which was a high value compared with the average hydrophobicity of peptides produced during haemoglobin hydrolysis by pepsin which is about 1000 cal/mol. Moreover, these haemorphins were present in some very complex peptidic mixtures which contained more than hundreds of peptides with different sequence [29].

Fig. 3 shows chromatographic profiles of the organic phases after ion-pair micro-extraction in the absence of alkyl-sulfonic acids at pH 3, 4, 5, 7 and 10 and for DH 3% (Fig. 3a) and DH 15% (Fig. 3b) hydrolysates respectively. Haem was extracted in the octan-1-ol phase and was detected at a retention time of 37 min: its quantity decreased when the aqueous pH increased. Haem structure contains carboxyl groups which were not ionized at acidic pH and therefore increased its hydrophobicity. However, when the aqueous phase was at basic pH, less haem was extracted because of the carboxyl groups ionization.

Few peptides were extracted at acidic aqueous phase pH for each hydrolysate, whereas the extracted peptide number increased when the pH value is upper or equal to 7. Among these peptides, LVVh-7 (RT about 29 min) and VVh-7 (RT about 28 min) were present in the organic phase for the respective studied 3% and 15% hydrolysates. Their increase could be explained by their high hydrophobicity.

In the following studies, it was supposed that the alkyl-sulfonic acid adding could mediate the haemorphin transport in the organic phase and also improve their extraction.

3.2. Effect of combined alkyl chain length and pH on haemorphin partitioning

The objective of this study was to determine the effect of the combination of alkyl chain length and aqueous pH on the haemorphin recovery by ion-pair extraction. Variation in extraction time and organic solvent polarity were not considered. However, preliminary studies have shown that there was no difference in extraction recovery between 6 and 24 h. At this stage, it was decided to investigate extraction recoveries and enrichment only by varying chemical parameters. This study allowed also to choose conditions to prepare LVVh-7 and VVh-7 by extraction assisted by ion-pairing with an alkyl-sulfonic acid.

Therefore, ion-pair extraction of haemorphins was carried out in a water/octan-1-ol biphasic system at room temperature and during 12 h. The aqueous phase contained the DH 3% or 15% hydrolysate and alkyl-sulfonic acid with different carbon numbers (6, 7 or 8), in the presence of 50 mM phosphate salt to maintain an ionic strength during the extraction. Although there are an almost unlimited number of possible carrier candidates, alkyl-sulfonic acids were chosen as they have proved their ion-pair mediated transport capability for cationic substances [30]. The pH of the aqueous phase varied from 3 to 10. It was emphasized that the amounts of alkyl-sulfonic acids employed in the experiments were well below the critical micelle concentration [31]; thus, the haemorphin extraction behaviour was distinct from that observed for micellar systems.

Fig. 4 shows an example of chromatographic profiles of organic phase after ion-pair micro-extraction at pH 3 on DH 3% (Fig. 4a) and 15% (Fig. 4b) hydrolysates respectively and with hexane, heptane or octane-sulfonic acid. It was observed that, for each tested surfactant agent, LVVh-7 and VVh-7 were extracted with the respective DH 3% and 15% hydrolysates (retention times of 27.5 min for LVVh-7 and 25.5 min for VVh-7). Haem was also extracted in the organic phases with a retention time of 37 min.

Chromatographic profiles, issuing from the experiments at different pH and with each surfactant agent, have allowed to evaluate the effect of the alkyl chain length/pH combination by the determination of the haemorphin recovery (R) and the haemorphin enrichment ratio (ER) in the organic phase, as it is explained in Section 2. Figs. 5 and 6 show the respective LVVh-7 and VVh-7 R (Figs. 5a and 6a) and ER (Figs. 5b and 6b) evolutions in the organic phase as a function of pH and in the presence of hexane, heptane or octane-sulfonic acid. The ER is an evaluation of the ion-pair micro-

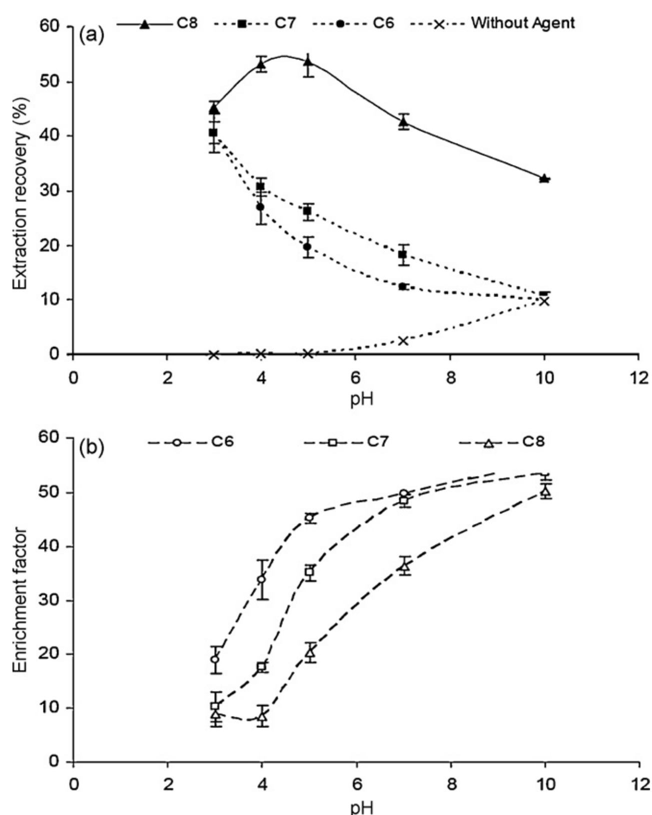

Fig. 5. LVVh-7 recovery (a) and enrichment ratio (b) by ion-pair micro-extraction in function of pH on a DH 3% peptic hydrolysate in a water/octan-1-ol biphasic system and in the presence of hexane, heptane and octane-sulfonic acid. All values are mean ± standard deviation based on three determinations.

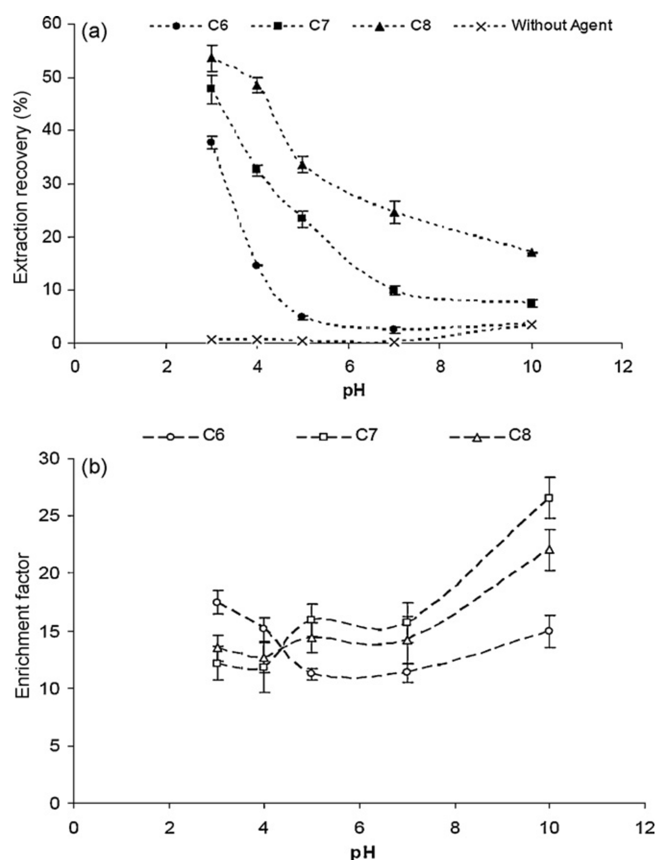

Fig. 6. VVh-7 recovery (a) and enrichment ratio (b) by ion-pair micro-extraction in function of pH on a DH 15% peptic hydrolysate in a water/octan-1-ol biphasic system and in the presence of hexane, heptane and octane-sulfonic acid. All values are mean ± standard deviation based on three determinations.

extraction system selectivity because it defined the purification degree of extracted haemorphin in the organic phase.

LVVh-7 and VVh-7 *R* evolution was compared with the experiments without surfactant agent. The alkyl-sulfonic acid adding influenced haemorphin extraction whatever the aqueous phase pH. It appeared that the octane-sulfonic acid allowed a better haemorphin extraction than the heptane and hexane-sulfonic acids from a pH value of 3, whereas a low difference was observed when the pH increased towards the value of 10.

Figs. 5b and 6b show that an ER was obtained for the two hydrolysates whatever the aqueous phase pH and the used alkyl-sulfonic acid. However, when the extraction occurred at acidic pH, a low haemorphin ER was obtained, due to the appearance of many other peptidic fractions in the octan-1-ol phase (see chromatographic profiles in Fig. 4). This peptidic fraction number was much reduced when the aqueous phase pH increased, due to the positive charge disappearance in the side-chain of arginine, lysine and histidine residues and the negative charge appearance in the side-chain of aspartic and glutamic acids present in the two haemorphins.

In the objective to determine the best conditions of haemorphin extraction, it was important to consider both the effect of the alkyl chain length/pH combination and the haem behaviour during the extraction, on the haemorphin *R* and ER responses. Therefore, the extraction occurred with the octane-sulfonic acid at pH 5 or 7 and with heptanes or octane-sulfonic acid at pH 5 or 7 appeared to be a good compromise for LVVh-7 and VVh-7 respectively.

A comparison of the haemorphin partition coefficients has been realized (Table 1) with alkyl-sulfonic acid in the best extraction conditions, without alkyl-sulfonic acid addition and with previous studies where the extraction occurred in an aqueous/butan-2-ol–octan-1-ol biphasic medium [24,25]. It is observed a high partition coefficient increased when the extraction occurred in the presence of the alkyl-sulfonic acid, with an improvement of about 40 times the values for each haemorphin compared with experiments without ion-pairing agent, and a 5 time improvement compared with the previous studies [24,25]. These comparisons demonstrated the effective extraction of the opioid peptides thanks to the alkyl-sulfonic agent using and the real potential of the peptide selective separation acquired with ion-pairing extraction in controlled liquid/liquid biphasic medium.

Table 1
Partition coefficients of the haemorphins with and without alkyl-sulfonic acid and in Refs. [24,25] conditions. Mean and standard deviation (S.D.) based on three determinations.

	Without agent	With agent	Refs. [24,25]
LVVh-7 partition coefficient	0.04 ± 0.01	1.64 ± 0.10	0.33
VVh-7 partition coefficient	0.03 ± 0.01	1.60 ± 0.15	0.31

4. Conclusion and perspectives

Ion-pairing concept was applied on peptic hydrolysates issue from bovine haemoglobin in a water/octan-1-ol biphasic system and in the presence of alkyl-sulfonic acids. This study allowed to show the feasibility of ion-pairing on LVVh-7 and VVh-7 extraction from complex peptidic hydrolysates. It has also showed that the alkyl chain length/aqueous phase pH combination, and the haem behaviour during extraction, had an effect on the haemorphin recovery and enrichment ratio.

The researches have shown that the conditions for optimal recovery varied from peptide to peptide. Therefore, the best conditions to extract LVVh-7, allowing convenient enrichment ratio and yield and the absence of haem precipitation, would be the use of octane-sulfonic acid at aqueous phase pH of 5 or 7. For VVh-7, the best conditions would be the use of the octane or the heptane-sulfonic acid and an aqueous phase pH of 5 or 7. In these conditions, the partition coefficient of each haemorphin has been improvement about 40 times compared to those without agents.

Separation of haemorphins by ion-pairing with amphipathic alkyl-sulfonic acids, in a liquid/liquid biphasic system and from bovine haemoglobin complex peptic hydrolysates, also appeared as an efficient alternative to the fractionation with chromatographic methods. Moreover, although there are many more conditions which might be tested (other negatively charged counter-ions or counter-ion concentration), it is obvious that there is a large potential in this system for selective extraction of peptides.

Recovery of extracted haemorphin by evaporation of the octan-1-ol appears to be very difficult, owing to the high boiling temperature of the solvent (about 195 °C). Previously, aluminium oxide support has allowed to haemorphin and haem separate in a butan-2-ol–octan-1-ol mixture [25]. This support could be tested to haemorphin recover in the octan-1-ol organic phase after ion-pairing extraction.

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