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Continuous production of a peptidic fraction containing the intermediate opioid peptide LVV-haemorphin-7 (LVVh-7) by peptic hydrolysis of bovine haemoglobin in a continuous membrane reactor

Romain Kapel, Renato Froidevaux, Naima Nedjar-Arroume, Anne Fertin-Bazus, Pascal Dhulster¹ and Didier Guillochon

Laboratoire de Technologie des Substances Naturelles, Bâtiment EUDIL/IAAL aile C, boulevard Paul Langevin, cité scientifique, 59655 Villeneuve d'Ascq, France

Peptic hydrolysis of native bovine haemoglobin at pH 3 yields the LVV-haemorphin-7 (Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe; LVVh-7) opioid peptide corresponding to the residues-31–40 fragment of the β -chain of haemoglobin. This peptide is intermediate in the course of batch hydrolysis and is rapidly degraded. Indeed, it shows an optimum at 3 % degree of hydrolysis (i.e. 2 min of reaction time). The hydrolysis was carried out in a continuous membrane reactor with a space time (ratio of the flux to the reactor volume) set to 2 min (corresponding to optimum LVVh-7 production). This process allows the continuous production of a constant fraction of intermediate peptides containing LVVh-7 for 48 min.

Introduction

For many years, enzymic hydrolysis of proteins has been used in the food industry, mainly to improve their functional properties, such as taste, solubility, emulsification or gelation [1]. Recently, peptides exhibiting biological activities such as antihypertensive [2], antimicrobial [3] or opioid [4] activities were isolated in hydrolysates of proteins, increasing the interest of enzymic proteolysis. Numerous peptides showing an opioid-like activity were isolated from hydrolysates of a wide range of animal or vegetable food protein such as β -casein [5], wheat gluten [6], soya proteins [7], whey protein [8] or spinach RuBisCO (ribulose biphosphate carboxylase/oxygenase) [9]. These new kinds of peptides were termed 'exorphins', in order to contrast them with the 'endorphins' released *in vivo* from endogenous precursors [10].

Bovine haemoglobin from slaughterhouse blood constitutes an important waste product and is a completely characterized protein. Workers have focused their studies on the hydrolysis of this protein in order to economically

exploit this by-product of the food industry [11,12]. Interestingly, many exorphins, called haemorphins, were isolated from bovine-haemoglobin hydrolysates [13]. Hence these haemorphins were intermediate peptides (IPs), generated at the beginning of proteolysis and rapidly degraded. Hydrolysates containing these peptides were then produced with difficulty.

We reported that LVV-haemorphin 7 (LVVh-7; Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe) was an intermediate opioid peptide corresponding to the sequence 31–40 of the β -chain of bovine haemoglobin [14]. Studies on kinetic appearance of this haemorphin in the course of peptic hydrolysis revealed that LVVh-7 was degraded particularly quickly. Indeed, the optimal concentration of this peptide was determined for 3 % degree of hydrolysis (DH), corresponding to a 2.5 min reaction time [15].

The continuous membrane reactor is a very convenient method of producing peptides. Indeed, it allowed us to obtain reproducible hydrolysates with increased productivity. Furthermore, haemoglobin hydrolysis carried out in a continuous membrane reactor generated decolorized hydrolysates, owing to high haem-retention rate [16]. However, most of these applications were to produce final peptides generated at the end of proteolysis. In the present paper we report on the continuous production, by a continuous membrane reactor, of a decolorized intermediate hydrolysate containing LVVh-7. The kinetic appearance of LVVh-7 has previously been reported in the course of a pH 4.5 peptic hydrolysis of haemoglobin denatured by urea, but this agent prevented scaling-up in continuous process.

Key words: decolorization, haemorphins, ultrafiltration.

Abbreviations used: CR, regenerated cellulose; DH, degree of hydrolysis; IP, intermediate peptide; LVVh-7, LVV-haemorphin-7 (Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe); MALDI-MS, matrix-assisted laser-desorption-ionization MS; MMCO, molecular-mass cut-off; RP-, reversed-phase.

¹ To whom correspondence should be addressed (e-mail pascal.dhulster@univ-lille1.fr).

So we developed a new peptic hydrolysis at pH 3, generating IPs and LVVh-7. Then a 3%-DH batch hydrolysate was ultrafiltered and IP transmission, haem retention and membrane fouling were investigated. Finally the peptic hydrolysis of bovine haemoglobin at pH 3 was carried out in a continuous stirred-tank membrane reactor.

Materials and methods

Materials

All common chemicals were of analytical grade and from commercial sources. Bovine haemoglobin, pig pepsin (EC 3.4.23.1, 40 000 Anson units) and pepstatin A were purchased from Sigma Chemicals. Acetonitrile was of HPLC grade. Water was obtained from a Culligan® (Northbrook, IL, U.S.A.) system; the resistivity was approx. 18 M Ω . All HPLC elutants were bubbled with helium.

Batch hydrolysis and ultrafiltration

Batch hydrolysis was carried out at pH 3 in a 100 ml-volume thermostatically controlled reactor (25 °C) containing 1% (w/v) haemoglobin and pig pepsin (EC 3.4.23.1) with an enzyme/substrate ratio of 2.5% (w/w). The reaction was stopped by the addition of pepstatin A. The hydrolysate (100 ml) was then ultrafiltered with a planar module (Minitan ultrafiltration system; Millipore Corporation) containing a hydrophilic regenerated cellulose (CR) membrane [catalogue no. PLGC OM PO4; 30 cm² surface area and 10 kDa molecular-mass cut-off (MMCO); Millipore]. The transmembrane pressure was set to 1 bar (0.1 MPa). The ultrafiltration was stopped when the volume of retentate had been decreased fivefold.

Determination of the DH of hemoglobin

The DH was defined as the ratio of the number of peptide bonds cleaved to the total number of peptide bonds in haemoglobin. The cleavage of peptide bonds during hydrolysis was quantified by the trinitrobenzenesulphonate method [17].

Continuous hydrolysis in an enzymic membrane reactor

The hydrolysis described above was carried out in a 40 ml continuous reactor thermostatically controlled at 25 °C coupled with a Minitan planar module of ultrafiltration containing 10 CR membranes. The pH was regulated in the course of the reaction by a pH-stat apparatus (Technical Brown). The reactor (40 ml) was opened after 2 min of hydrolysis. The 2 min space time chosen was obtained by holding the filtrate flow at

20 ml/min. This filtrate flow itself was maintained by raising the transmembrane pressure.

Sample preparation for reversed-phase (RP)-HPLC analysis

The batch or continuous filtrate samples were directly injected into the RP-HPLC system. The batch and continuous retentate samples were diluted prior injection in order for them to be compared. Then batch retentate samples were all diluted fivefold so that they could be compared with the filtrate. Indeed, the batch ultrafiltration was stopped when the volume of retentate had been decreased fivefold (that is, corresponding to a fivefold concentration). The continuous retentates were taken after passage of 1, 8, 17 and 23 reactor volumes, corresponding to concentrations of 2-, 9-, 18- and 24-fold. These samples then had to be diluted 1:2, 1:9, 1:18 and 1:24.

RP-HPLC system

The liquid-chromatographic system consisted of a Waters TM 600 automated gradient controller pump module, a Water Wisp 717 automatic sampling device and a Waters 996 photo-diode-array detector. Spectral and chromatographic data were stored on an NEC image 446 computer. Millennium software was used to plot, acquire and analyse chromatographic data. All the chromatographic processes were performed with a Vydac C₄ column (250 mm \times 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 vol.) as eluent B. The flow rate was 1 ml/min. Samples were filtered through 0.22- μ m-pore-size filters prior to injection. The gradient applied was 0–67% (v/v) eluant B over 30 min, followed by 67–87% (v/v) eluant B over 35 min. Online UV-absorbance scans were performed at 226 nm. Chromatographic analyses were completed with Millennium software.

Matrix-assisted laser-desorption-ionization (MALDI)-MS

MALDI-MS was performed on a Finnigan Mat Vision 2000 time-of-flight mass spectrometer equipped with a viewing unit. Data were acquired at 500 MHz sampling frequency in reflector mode with external and/or internal calibration and also in linear mode. The nitrogen laser spot size was 70 μ m and the laser energy was adjusted close to threshold. The following matrix was used: a mixture of 2,5-dihydroxybenzoic acid (12 g/l) and 2-hydroxy-5-methoxybenzoic acid (12 g/l) (9:1, v/v) was prepared with acetonitrile/water (1:2, v/v) as solvent. A 1 μ l portion of the matrix solution was mixed with 0.5 μ l of sample solution

on a stainless-steel target, dried in a gentle stream of air and loaded into the mass spectrometer. Mass spectra were obtained preferably from the crystalline rim of the dried sample. Usually 15–30 spectra were averaged in linear mode and 20–40 in reflectron mode. Linear configuration was used, applying 30 kEv ion energy for reflectron time-of-flight analyser 5 kEv ion energy and 20 kEv post acceleration to a discrete dynode secondary electron multiplier in positive-ion mode.

Results and discussion

Detection of LVVh-7 from an haemoglobin peptic hydrolysate obtained at pH 3

The peptic hydrolysis of native haemoglobin was performed for 20 min at 25 °C at pH 3. The resulting hydrolysates were analysed in one step by RP-HPLC on a C₄ column according to the method previously described [14]. The chromatograms of hydrolysates obtained at 2, 10 and 20 min under these conditions, and bovine haemoglobin (1%, w/w) as a control, are shown in Figure 1. The fractions eluted after haem (40 min) were identified as the α and β chains of bovine haemoglobin. Only residues of haemoglobin chains were detected after 2 min hydrolysis. The whole haemoglobin chains were then rapidly hydrolysed into peptides. Most of the peptides between 30 and 37 min generated at 2 min hydrolysis were themselves hydrolysed after 10 min, and only few of them still remained after 20 min. These peptides were then intermediate in the course of hydrolysis and termed 'IP' (for IPs) on the Figure.

The fraction with retention time of 29.7 min was recognized as LVVh-7. This peptide followed the kinetics of the IPs described above, showing an optimum at 2 min of hydrolysis, corresponding to 3% DH.

The positive MALDI spectra of this fraction at 3% DH are shown in Figure 2. The accurate relative molecular mass of the main peptide, deduced from the m/z value of $(M+H^+)$, was 1308 Da. On the basis of these results and taking into account the known amino acid sequence of the α - and β -chains of bovine haemoglobin, this peptide corresponded to the residues 31–40 of the β chain:

Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe

that is, LVVh-7.

These results showed that the peptic proteolytic mechanism of native bovine haemoglobin at pH 3 was 'zipper' rather than 'one-by-one' in nature. In the course of hydrolysis, IPs ('IP'), including LVVh-7, were rapidly generated and degraded. Furthermore an optimum of LVVh-7 concentration was observed for very low DH (around 3%). Similar results were previously reported in the course of peptic hydrolysis at pH 4.5 [14], but under these conditions haemoglobin had to be denatured by urea,

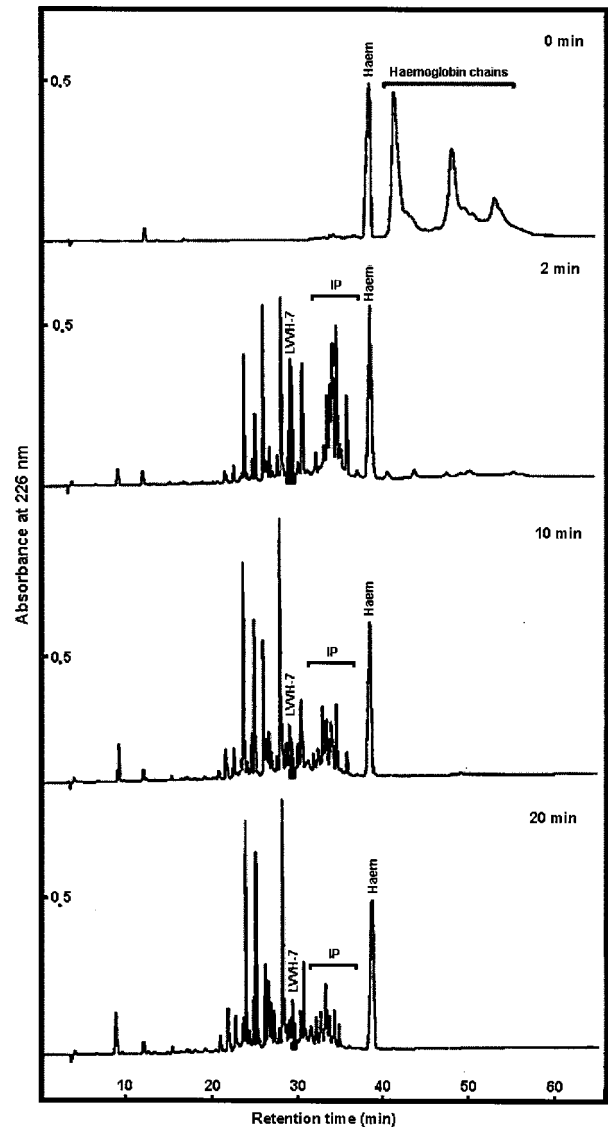


Figure 1 HPLC chromatogram of haemoglobin hydrolysate at different times (initial, 2 min, 10 min and 20 min)

The peptic hydrolysis was performed at pH 3 and at a temperature of 25 °C. Hydrolysates were analysed by RP-HPLC on a C₄ column with the conditions described in the Materials and methods section.

precluding scaling-up in continuous process (urea is an inconvenient reagent for food application).

Batch-hydrolysate ultrafiltration

The ultrafiltration of the 3% DH batch hydrolysate with a hydrophilic 10-kDa-MMCO CR membrane (CR 10) was then investigated. Indeed the 10 kDa MMCO membrane was commonly used for peptide ultrafiltration. Furthermore, hydrophilic membranes have been reported to prevent irreversible fouling – a phenomenon considered as the major limiting factor in protein ultrafiltration. The ultrafiltration

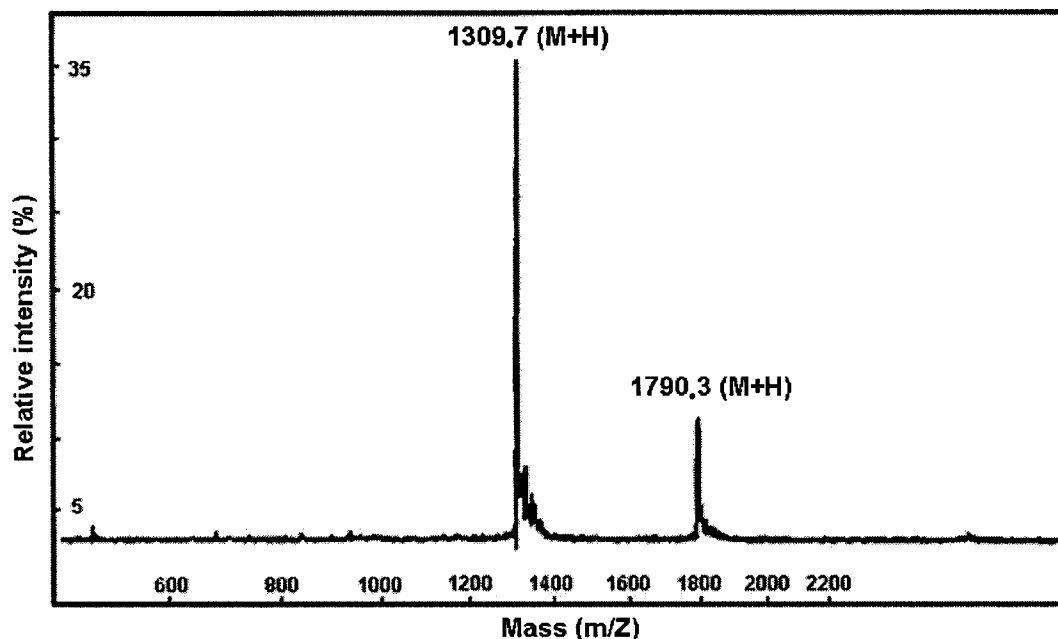


Figure 2 MALDI-MS spectra of LVVh-7 fraction isolated by HPLC

LVVh-7 corresponds to residues 31–40 of the β -chain of haemoglobin (i.e. Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe).

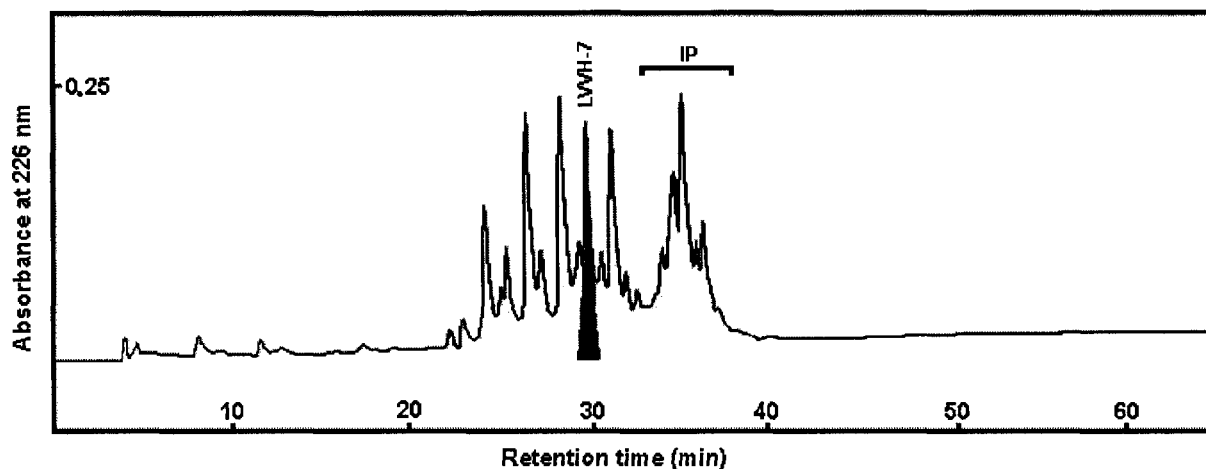


Figure 3 HPLC chromatogram of the permeate obtained after ultrafiltration of the 3%-DH hydrolysate with the 10 kDa-MMCO CR membrane

The 3% batch hydrolysate was ultrafiltered through a 10 kDa MWCO CR membrane under a 1 bar transmembrane pressure. The ultrafiltration process was stopped when the retentate was fivefold reduced.

process was carried out at pH 3 under 1 bar of transmembrane pressure and stopped when the retentate volume had been decreased fivefold.

A Kjeldahl protein analysis of both filtrate and retentate allowed us to determine a good (57%) peptide transmission rate (ratio of peptide concentration in the filtrate to that in the retentate) after the batch ultrafiltration. Furthermore the filtrate was analysed by HPLC, as shown in Figure 3. The

IP fraction and LVVh-7 can be seen to have passed successfully through the membrane, and no haem peak was detected.

The standard water flux of the membrane at 1 bar transmembrane pressure was also investigated to study the irreversible fouling of CR 10 caused by ultrafiltration of the 3%-DH hydrolysate. Interestingly, no differences of standard water flux before and after ultrafiltration were observed.

These results indicate that no irreversible fouling of the CR 10 membrane was generated by the 3%-DH batch hydrolysate ultrafiltration process. Moreover, the filtrate contained the IPs, including LVVh-7, and was completely decolorized (that is, haem-free). The CR material selected was also convenient for continuous production of the intermediate 3%-DH hydrolysate.

Continuous production of IPs containing LVVh-7 in enzymic membrane reactor

The peptic hydrolysis of native haemoglobin was carried out at pH 3 in a 40 ml continuous membrane reactor for 48 min. The reactor was opened after 2 min of batch hydrolysis and the space time was set at 2 min, since this reaction time was convenient to produce IPs and LVVh-7 in the course of batch hydrolysis. According to the definition of space time:

$$\text{Space time} = \text{flux } (J) / \text{reactor volume } (V)$$

the permeate flux was 20 ml/min. This permeate flux was kept constant, thus causing the transmembrane pressure to rise. The evolution of the transmembrane pressure and permeate flux is reported in Figure 4. At the start of continuous process, the 20 ml/min flow rate rose when 1 bar transmembrane pressure was applied. This flux was kept constant by increasing the transmembrane pressure up to 2.6 bar (0.26 MPa) (after 34 min hydrolysis time). Above 2.6 bar, the permeate flux proved difficult to regulate, and the continuous enzymic membrane reactor was stopped when the transmembrane pressure rose to 3.2 bar (0.32 MPa) (after 48 min of hydrolysis time or 46 min of continuous process). This range of transmembrane pressure allowed a permeate flux between 17 and 19 ml/min. During this period the space time was then slightly longer than 2 min. The limit of 3.2 bar transmembrane pressure determined the stability of the continuous reactor with our ultrafiltration system under these conditions. Generally, the literature reports that the irreversible adsorption of molecules on the membrane surface, or into the pores, generally stops the continuous reactor membrane processes. Interestingly, in our case, the water permeate flux was recoverable after continuous process without washing the membrane. These results show that the stability of this continuous reactor was essentially limited by the resistance of our ultrafiltration system and not by a real fouling of the membrane.

From the opening of reactor (after the 2 min of batch hydrolysis), the permeate and retentate were analysed by HPLC every 2 min – a period of time corresponding to the filtration of one reactor volume. The retentate and filtrate HPLC profiles after the first, eighth, sixteenth and last reactor volume passage, respectively corresponding to 4, 18, 34 and 48 min of hydrolysis are presented in Figure 5. From

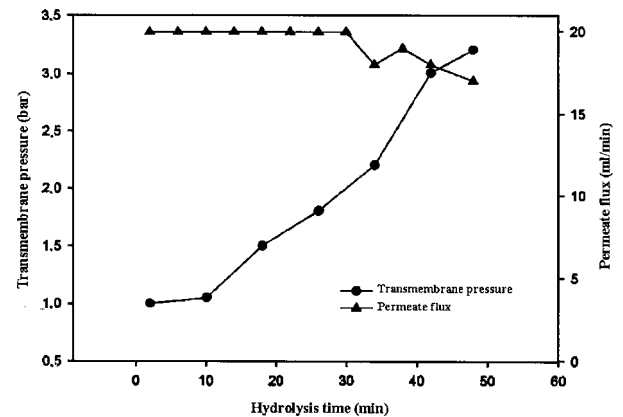


Figure 4 Transmembrane differential pressure evolution and filtrate flux evolution versus time in course of the hydrolysis carried out in the continuous membrane reactor

4 to 18 min of continuous hydrolysis, slight differences in retentate profiles were observed, but the IP fraction, which was eluted between 30 and 35 min of retention time, and LVVh-7, characteristic of a low DH, were recognized. Only haemoglobin chain residues were detected in both (4 min and 18 min) retentates. From 18 to 34 min, no changes in the peptide profiles were seen. Then, from 34 to 48 min, the IP fraction and haemoglobin chains decreased a little. However, these IPs and LVVh-7 were still detected. So 18 min were necessary to establish a stationary step that was held up to 24 min. During this stationary step, IPs and LVVh-7 were continuously generated, a low DH being held in the continuous reactor. As a matter of fact, under the hydrolysis conditions following a zipper-type mechanism, haemoglobin is hydrolysed much more quickly than the IPs are generated, due to a greater affinity of pepsin for the protein. In our system, the retentate was continuously fed with haemoglobin, and a portion of IPs was continuously extracted in filtrate. So the space time applied allowed the haemoglobin to be preferentially hydrolysed compared with IPs, despite their accumulation in the retentate. From 34 to 48 min of hydrolysis the proportion of IPs and haemoglobin chains decreased with regard to the proportion of smaller peptides eluted from 20 to 30 min of retention time. During this period, an increase of space time occurred, caused by decrease of permeate flow, thus explaining the decrease in the proportion of haemoglobin. Furthermore, the concentration of IPs increased continuously from the start of process, because of their retention by the ultrafiltration steps. So, under these conditions, a significant hydrolysis of IPs occurred, despite the better affinity of pepsin for haemoglobin. However, IPs and LVVh-7, which were rapidly degraded in the course of batch hydrolysis (as shown in Figure 1), were generated for 48 min when

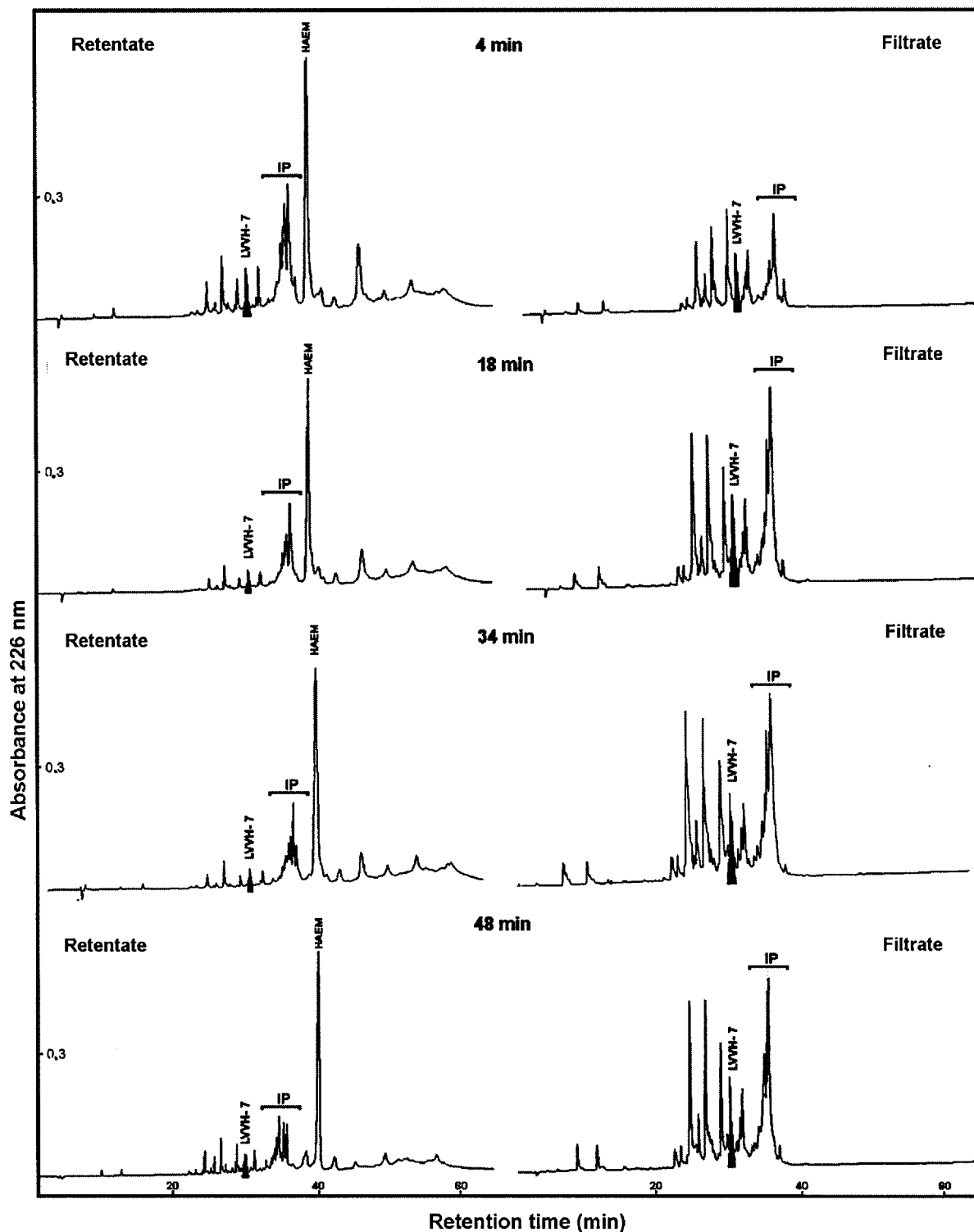


Figure 5 HPLC chromatograms of retentate and filtrate obtained at 4, 18, 34 and 48 min of continuous hydrolysis, corresponding respectively to passages of 1, 8, 16 and 23 reactor volumes

The retentates were respectively diluted 1:2, 1:9, 1:17 and 1:24 in order that they might be compared.

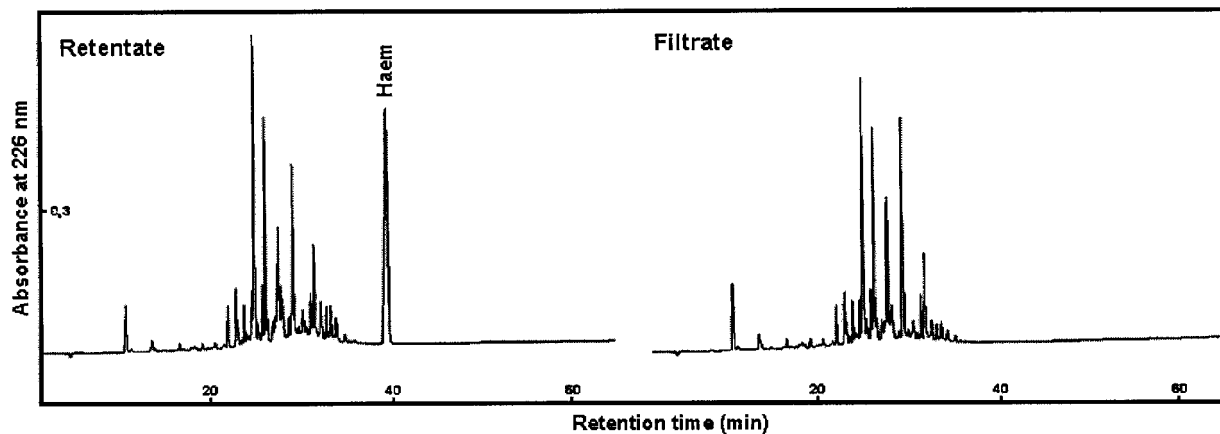


Figure 6 HPLC chromatograms of retentate and filtrate obtained after ultrafiltration of the 48 min batch hydrolysate

The retentate was diluted 1:5 in order that it might be compared with the filtrate (indeed the ultrafiltration was stopped when retentate volume had been decreased fivefold).

the hydrolysis was carried out in the continuous membrane reactor.

To establish the stationary step (between 4 and 18 min), the filtrate profiles changed a little, but the IP fraction and LVVh-7 were still extracted and haem was totally retained. Interestingly, no modification in the filtrate profiles was noticed from 18 min until the end of the process. This result indicates that the same peptides, including IPs and LVVh-7, passed through the membrane at each ultrafiltration step, despite the slight change in retentate at the end of process.

A 48 min batch hydrolysate was ultrafiltered as a control. The HPLC chromatogram of retentate and filtrate are shown in Figure 6. As expected, the 48 min batch retentate and filtrate profiles are totally different from those of the 48 min continuous retentate and filtrate. Indeed, in neither the retentate nor the filtrate were IPs, LVVh-7 and haemoglobin chain residues detected. Furthermore, as the fraction of IPs (eluted between 30 and 35 min of retention time) decreased, the fraction of smaller peptides (eluted between 20 and 30 min) was increased. This result indicates that, during 48 min of batch hydrolysis, IPs and LVVh-7 were totally hydrolysed into smaller peptides and did not appear in the filtrate. So the peptic hydrolysis of native haemoglobin carried out in our continuous membrane reactor allowed the continuous extraction of IPs containing LVVh-7 for 48 min. Indeed, the continuous mode seems to have held a low DH in the system, preventing the hydrolysis of IPs.

The continuous process allowed us to generate a constant decolorized fraction of IPs containing LVVh-7 during 48 min, and 920 ml of filtrate (i.e. 23 reactor volumes) were produced. Such a product would have been produced after ultrafiltration of a batch hydrolysate stopped at 3% DH

(i.e. 2 min of hydrolysis time), but the quantity of enzyme used would be 23-fold increased and a peptic inhibitor would have to be used. Furthermore, our system could easily be scaled up, increasing the volume of the reactor and the membrane area, in order to get a filtrate flux allowing a space time of 2 min. Moreover, membrane fouling is the major limiting factor, generally leading to the end of use of a continuous enzymic membrane reactor, and we showed that the end of our process was due rather to the mechanical resistance of our laboratory-scale ultrafiltration system. The enzymic membrane reactor could then work much longer than 48 min using a higher-scale ultrafiltration system.

Conclusion

The purpose of the present study was to investigate the continuous production of a fraction of IPs containing the opioid peptide LVVh-7 in a membrane reactor. We have shown here that an enzymic membrane reactor allowed us to produce a continuous constant permeate containing IPs, including LVVh-7. Moreover, the permeate was completely decolorized. These peptides were obtained from a hydrolysis of native haemoglobin bovine at pH 3, carried out in the continuous membrane reactor for 48 min and which corresponded to the ultrafiltration of 23 reactor volumes. The advantages of this process were the low quantity of enzyme used and an easy scale-up. Furthermore, an increasing of the working time of our membrane reactor could be expected using a stronger ultrafiltration system.

The fraction produced could be potentially considered as a final product, since such a fraction, containing an opioid peptide from milk protein, is commercially available as a fraction with stress-relieving properties (PRODIET F 200; Ingredia, Arras, France). Further haemorphin purifi-

cation is also possible. Indeed, an extraction process, developed in our laboratory, combining hydrophobic and ion-exchange chromatography, permitted us to obtain pure haemorphins from a complex peptic haemoglobin hydrolysate [18]. Our decolorized filtrate could be very convenient for this purification process, since haem disturbs hydrophobic chromatography. However, chromatography processes involve high costs and are time-consuming. Recently, LVVh-7 extractions from complex hydrolysates have successfully been achieved by the use of solvents such as butan-2-ol [19] or a water/butan-2-ol/octan-1-ol mixture [20]. Furthermore, some workers have employed coupled solvent molecule extraction to membrane-reactor production [21]. So we envisage that it may be possible to couple solvent extraction of LVVh-7 with a continuous membrane reactor in order to get a continuous production of an LVVh-7-enriched peptide fraction.

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