Proteolytic degradation of hemoglobin by endogenous lysosomal proteases gives rise to bioactive peptides: hemorphins

Ingrid Fruitier[a], Isabelle Garreau[a,*], André Lacroix[b], Anny Cupo[c], Jean Marie Piot[a]

[a] *Laboratoire de Génie Protéique et Cellulaire, UPRES 2001, UFR Sciences, Université La Rochelle, Avenue Marillac, 17042 La Rochelle, France*
[b] *Centre d'Etudes Biologiques de Chizé CNRS-UPR 4701, 79360 Beauvoir-Sur-Niort, France*
[c] *Institut de Pharmacologie Moléculaire et Cellulaire CNRS-UPR 411, 660 Route des Lucioles, Sophia Antipolis, 06560 Valbonne, France*

Received 11 January 1999

Abstract Hemorphin generation by mice peritoneal macrophages has been recently reported, nevertheless no conclusive data exist to localize clearly the macrophage proteolytic activity implicated in their generation. Because lysosomes are believed to be the main site of degradation in the endocytic pathway, we have studied their potential implication in the generation of hemorphins from hemoglobin. When this protein is submitted to purified rat liver lysosomes, an early generation of hemorphin-7-related peptides, detected by a radioimmunoassay, was observed. These peptides seemed to be relatively stable during the first hours of hydrolysis.

Key words: Lysosome; Hemoglobin; Proteolysis; Hemorphin; Radioimmunoassay; HPLC

1. Introduction

In 1986, Brantl et al. [1] detected from a proteolytic treatment of hemoglobin a series of peptides, with an opioid-like activity, called hemorphins. This study was of great importance because it followed many other previous discoveries of biologically active peptides reproducing amino acid sequences of α- or β-globin chains [2]. Among these peptides, the best known, kyotorphin and neokyotorphin [3], corresponded respectively to the 140–141 and 137–141 C-terminal sequences of α-globin. Since the findings of Brantl et al., systematic studies of peptide composition of various tissue extracts resulted in a great number of new peptides with structure mainly originating from α- or β-globin chains [2]. In addition, some of these hemoglobin-derived peptides were often found to coexist with various pathologies such as human lung adenocarcinoma [4], Alzheimer's disease [5], Hodgkin's disease [6] and brain ischemia [7] and even subsequently to physical exercise like long distance running [8]. In the latter study, it was noteworthy that the plasma level of the released hemorphin-7 exceeded that of β-endorphins by a factor of about 1000. Finally, an attractive feature of the hemorphins is their link to the β-endorphin system: the hemoglobin-derived opioid peptides have the capacity to release the classical opioid peptides from the pituitary into the circulatory system [9].

It was then becoming clear that hemoglobin, besides its primordial function as an oxygen carrier, could serve in vivo as a powerful source of bioactive peptides playing a profound role in homeostasis [2]. This assessment could be probably closely connected with a suggestion of Carraway et al. [10] about the existence of a putative endogenous processing which generates neurotensin and enkephalin-related peptides in blood circulation from plasma proteins. The main question arising from the above-mentioned considerations concerns the possible way of natural generation of hemorphins in the organism.

In a previous study [11], two opioid peptides, corresponding to the sequence 32–40 (VV-hemorphin-7) and 31–40 (LVV-hemorphin-7) of the β-chain of bovine hemoglobin, were isolated in vitro from a bovine hemoglobin peptic hydrolysate. According to the fundamental knowledge of the physiology of macrophages and their role in the cellular protein catabolism and/or in the inflammatory process, we have investigated the appearance of hemorphins when hemoglobin is in the presence of mice peritoneal macrophages [12]. It was then clearly demonstrated that under these conditions, VV-hemorphin-7 could be released from globin. Nevertheless, no conclusive data exist to localize clearly the origin of the proteolytic activity responsible for hemorphin release, even if subsequent studies suggested that hemorphin generation could be the result of an acidic protease cleavage [12,13].

The aim of the present study was to investigate the involvement of the macrophage proteolytic complexes in hemorphin generation. For this purpose, rat hepatic lysosomes were purified in order to expose directly hemoglobin to lysosomal enzymes. Moreover, analytical tools previously developed in our laboratory to identify peptides from complex proteolytic mixtures have been applied: reversed phase high performance liquid chromatography (RP-HPLC) on-line with spectral scan analysis [14], amino acid analysis [15] and mass spectrometry analysis [16]. A specific radioimmunoassay (RIA) against the C-terminal part of VV-hemorphin-7, recently perfected and validated [17] was also performed to verify the patterns of generated hemorphins.

2. Materials and methods

2.1. Materials and chemicals

HPLC analyses were performed with a Waters 600 automated gradient controller-pump module, a Waters wisp 717 automatic sampling device and a Waters 996 photodiode array detector (Milford, MA, USA). Deltapak C_{18} column was purchased from Waters (Milford, MA, USA). The Millenium software was used to plot, acquire and treat spectral and chromatographic data.

Trisodium citrate was from Prolabo and sucrose from Acros Or-

*Corresponding author. Fax: +33 5-46-45-82-47.
E-mail: igarreau@univ-lr.fr

This work is a part of the Ph.D. thesis of I. Fruitier under the supervision of I. Garreau and J.M. Piot.

PII: S0014-5793(99)00271-9

ganics. HEPES, bovine serum albumin (BSA) and metrizamide (centrifugation grade) were from Sigma. Picotag sample diluent and eluent A were from Waters (Millipore Corporation, Milford, MA, USA). Amino acid standard H and phenyl isothiocyanate (PITC) were from Pierce (Rockford, IL, USA). Acetonitrile was of HPLC grade and aqueous HPLC eluents were degassed with helium during analysis. LVV-hemorphin-7, VV-hemorphin-6, VV-hemorphin-7 and hemorphin-7 were synthesized by Altergen (Schiltigheim, France). Prior to use, synthetic peptides were purified by HPLC. Synthetic VV-hemorphin-7 was iodinated according to the Hunter and Greenwood procedure [18]. Chemicals and solvents were of the best analytical quality available.

2.2. Methods

2.2.1. Preparation of rat liver lysosomal fractions. Lysosomes were obtained from rat liver as previously described [19,20]. Briefly, male Wistar rats (200–250 g), starved overnight, were anesthetized with chloroform and decapitated. The livers were immediately removed, chilled in ice-cold 0.25 M sucrose, and homogenized in the same medium by means of a Teflon pestle in a Potter Thomas tissue grinder. The homogenate was made up to a volume of 7 ml/g of liver and filtered on two layers of cheesecloth. After the centrifugation of the filtrate ($2500\times g$, 10 min, 4°C), the pellet (C_1) was discarded and the remaining supernatant (S_1) was centrifuged at $10\,000\times g$ for 20 min at 4°C. The resulting pellet was suspended in sucrose and centrifuged as previously. The final pellet (C_2) was loaded on the bottom of a discontinuous metrizamide gradient (adjusted to pH 7.0 with NaOH) and centrifuged 2 h at $95\,000\times g$ at 4°C (Beckman ultracentrifuge, rotor SW 28.1). Five different fractions were obtained: the 19.8–24.5 (F_1), 24.5–26.3 (F_2), 26.3–32.8 (F_3) metrizamide interfaces and the bottom layer named F_5. The fraction F_4 corresponds to the 32.8% metrizamide solution. The fractions were collected from the gradient, diluted five times with 0.25 M sucrose and centrifuged at $37\,000\times g$ for 10 min in the Beckman centrifuge (rotor SW 28.1). Pellets were then resuspended in a small volume of 0.1 M phosphate buffer, pH 7.3 and stored at −20°C until use.

Small aliquots of homogenate, pellets, supernatants and selected fractions of the metrizamide gradient were kept for both mitochondrial and lysosomal enzymes and protein content determinations. Qualitative evaluation of succinic dehydrogenase, a mitochondrial marker, and the degree of purification of lysosomes, estimated by the specific acid phosphatase activity in the fraction relative to the original homogenate, were estimated according to Tsou et al. [21]. The protein contents were determined by the Lowry procedure [22].

2.2.2. Hemoglobin proteolysis. Bovine hemoglobin was prepared from erythrocytes as described previously [17]. Lysosomes (200 μg of protein) from fraction F_3 were incubated with hemoglobin (0.2% w/v) in a final volume of 500 μl of 4 mM sodium citrate buffer, pH 4.5 at 37°C. Before the incubation, lysosomes were disrupted by freezing and thawing six times (broken lysosomes). Incubations were achieved during 1, 5, 10, 30, 60, 120 and 240 min.

The reactions were stopped by boiling incubation mixtures during 20 min. Residual hemoglobin was discarded by centrifugation (5000 rpm, 15 min), supernatants were collected by filtration using a 0.22 μm pore membrane and stored at −20°C until their chromatographic analysis. Two controls were carried out: hemoglobin alone and lysosomes alone were submitted to the same procedures of incubation and analysis.

2.2.3. Chromatographic and spectral scan analysis procedures. In order to characterize VV-hemorphin-7 immunoreactive peptides, supernatants of hemoglobin hydrolysates were separated by RP-HPLC using a Deltapak C_{18} column (300×3.9 mm i.d.) with a linear gradient of acetonitrile (eluent A: 10 mM ammonium acetate buffer, pH 6.0; eluent B: acetonitrile) from 15% to 40% B for 30 min after an initial isocratic step (15% B) over a period of 5 min. The flow rate was 1 ml/min. Aliquots (100 μl) of each supernatant from 1 to 240 min incubation were injected through the HPLC column. Fractions (1 ml each) were automatically collected, lyophilized and reconstituted in RIA buffer (HEPES buffer) in order to be assayed by radioimmunoassay.

Continuous UV absorbance spectral scans were performed, between 190 and 300 nm, during chromatographic acquisition. Spectrum matching results (i.e. comparison between spectra of each eluted peak and a library spectrum of hemorphins) were expressed as match angle (MA) and match threshold (MT). Spectral scan procedure has been extensively used in previous studies [13,14].

2.2.4. Determination of VV-hemorphin-7 peptides by radioimmunoassay. Standard, samples, tracer and antiserum were diluted in RIA buffer. Radioimmunoassay procedure for VV-hemorphin-7 was based on the precipitation with polyethylene glycol (PEG) and was conducted as described in a previous paper [17]. Briefly, the fractions from the RP-HPLC stage were lyophilized and reconstituted in 1 ml of RIA buffer. A 100 μl aliquot of the fraction or standard, 100 μl IG-C_2 antiserum (final dilution 1/125 000) and 100 μl iodinated peptide (5000 cpm) were incubated for 40 h at 4°C. After precipitation with PEG, the radioactivity of the pellet was measured and the bound/total ratio (B/T) calculated.

2.2.5. Amino acid analysis. Amino acids were analyzed using a Waters Picotag Work Station. Peptide hydrolysis was achieved with constant boiling HCl containing 1% phenol, for 24 h at 100°C. Pre-column derivatization of amino acids with phenyl isothiocyanate and HPLC separation of derivatized amino acids on a Waters RP-Picotag column (150×3.9 mm i.d.) were performed according to Bidlingmeyer et al. [23]. The flow rate was 1 ml/min and the detection was carried out at 254 nm.

2.2.6. MALDI (matrix assisted laser desorption ionization) mass spectrometry analysis. MALDI mass spectra were measured on a reflectron-type Vision 2000 time-of-flight mass spectrometer (Finnigan MAT, Bremen, Germany). Samples were mounted on an *x–y* moveable stage, allowing irradiation of selected sample areas. A nitrogen laser with an emission wavelength of 337 nm and a pulse duration of 3 ns was used. Spectra were recorded in the positive ion mode and accelerated to an energy of 5 keV before entering the flight tube. Ions were post-accelerated, for detection, to an energy of 5 keV. Samples were prepared by mixing, directly on the target, 1 μl of the peptides diluted to a concentration of 2–8 pmoles/μl and 1 μl of 2,5-dihydroxybenzoic acid (DHB) matrix solution (12 mg/ml dissolved in CH_3OH-H_2O (70:30)). The samples were allowed to dry for 3–4 min at room temperature. Electrospray mass spectra were acquired on a Micromass Quattro II triple-quadrapole mass spectrometer operating with an API ion source in positive ion electrospray mode. Peptide samples were diluted into acetonitrile/0.2% formic acid (1:1 by volume), to a concentration of 10 pM/μl and infused at 3 μl/min. Spectra were scanned with a cone voltage of 35 V.

Table 1
Protein content and activities of a lysosomal enzyme in homogenate, pellet C_2 from rat liver and in different fractions (F_1–F_5) obtained after centrifugation of C_2 in a discontinuous metrizamide gradient

	Protein (g)	Acid phosphatase (units/mg protein)	Purification degree[a]	Yield[b]
Homogenate	7.78	0.0603		
C_2	0.882	0.177		
F_1	0.0037	0.657	10.9	0.5
F_2	0.0045	1.116	18.5	1.1
F_3	0.0217	0.863	14.31	4
F_4	0.01346	0.7055	11.7	2
F_5	0.757	0.0467	0.774	7.5

[a]Results are given in relative specific activity, i.e. specific activity found in the fraction/specific activity measured in the homogenate.
[b]Results are given in total activity, i.e. total activity found in the fraction/total activity measured in the homogenate.

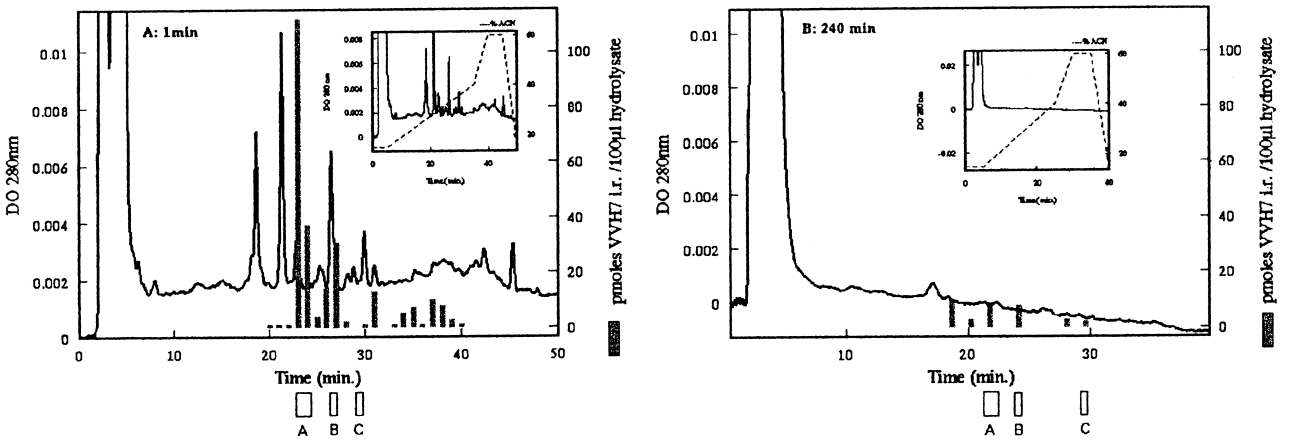

Fig. 1. UV absorbance profiles (280 nm) of HPLC chromatogram from hemoglobin hydrolysates. One hundred µl of hydrolysate corresponding to 1 (A) and 240 (B) min incubation, were applied to a Deltapak C_{18} column as described in Section 2. VV-hemorphin-7 content was determined and expressed as pmol of VVH7-i.r. per 100 µl of hydrolysate.

3. Results

In order to assess the role of lysosomes in the generation of hemorphins from hemoglobin, they were prepared from rat liver according to the procedure of Wattiaux et al. [19]. As estimated by the acid phosphatase activity, lysosomes were markedly purified in fractions F_1, F_2, F_3 and F_4 of the metrizamide gradient (Table 1). The degree of purification of lysosomes (based on acid phosphatase activity) recovered in fraction F_3, relative to the original homogenate was 14.31-fold and the yield 4% (total activity in the fraction/total activity in the homogenate). Relative to the fraction F_2, which exhibited the better specific activity (1.116), fraction F_3 constituted the best compromise between both a good specific activity (0.863) and yield (4% vs. 1.1% for F_2). The qualitative evaluation of the mitochondrial marker demonstrated that F_3 was free of mitochondria (data not shown).

In order to determine if a possible way of degradation of hemoglobin by macrophages could take place in lysosomes [12], we have submitted hemoglobin to broken lysosomes from fraction F_3. A kinetics of degradation of hemoglobin was carried out. The resulting hydrolysates were analyzed by RP-HPLC and collected fractions (1 ml) were assayed for their content in VV-hemorphin-7 by RIA. Chromatograms from proteolysis of hemoglobin after 1 and 240 min of incubation are displayed in Fig. 1. As soon as 1 min of hydrolysis (Fig. 1A), VV-hemorphin-7-related immunoreactive material (VVH7-i.r.) is released. The immunoreactive material was mainly detected in three fractions, named fraction A, fraction B and fraction C. The weak immunoreactivity detected between 32 and 40 min has not been precisely studied and could correspond to N-terminal extended hemorphin-7-related peptides previously described by Glämsta et al. [24]. After 240 min of hydrolysis (Fig. 1B), few amounts of peptides were detected at 280 nm and a very low VVH7-i.r. was observed in fractions A, B and C.

Immunoreactive material detected in the hydrolysate was undoubtedly the result of the co-incubation of hemoglobin with lysosomes since when both hemoglobin and/or lysosomes were incubated alone, no immunoreactive material was released whatever the incubation time (data not shown).

The level of VVH7-i.r. in fractions A, B and C during hydrolysis is presented in Fig. 2. The VVH7-i.r. found in fraction A increased rapidly, reached a maximum at 5 min and began to decrease after 60 min. After 240 min of incubation, a very small amount of VVH7-i.r. was detected. The same evolution was observed for the other two fractions (B and C), nevertheless, the immunoreactive material present in these fractions was always much lower than in fraction A.

Synthetic hemorphins were loaded on the RP-HPLC column, and their chromatographic parameters (retention time, MA and MT) compared with those obtained for fractions A, B and C (Table 2). According to this comparative study, four hemorphins were suspected: hemorphin-7 or VV-hemorphin-6

Table 2
Chromatographic parameters (retention time and spectra matching results) of immunoreactive fractions from hemoglobin after 10 min of incubation with lysosomal enzymes

Immunoreactive fractions	Spectral analysis[a]			Retention time (min) of synthetic peptide
	MA	MT	Match spectral result[b]	
Fraction A: 22–24 min	0.779	1.503	H7	22.983
	1.221	1.824	VVH6	23.188
Fraction B: 26–27 min	5.066	1.549	VVH7	26.588
Fraction C: 29–30 min	1.332	1.674	LVVH7	29.602

[a]Spectral analyses were carried out on synthetic peptides purified under the same chromatographic conditions.
[b]Deduced from Millenium software after UV comparison with a library spectrum of hemorphins.
MA, match angle; MT, match threshold [18].

in fraction A, VV-hemorphin-7 in fraction B and LVV-hemorphin-7 in fraction C. Concerning fraction B, UV spectral comparison designed actually VV-hemorphin-7 as the VVH7-i.r. Although the matching result obtained is not optimum (MA > MT) [14], we could consider the presence of VV-hemorphin-7 in this fraction since the only peptide eluted at such retention time and recognized by IG-C_2 antibodies is VV-hemorphin-7 [17]. The material eluted in fraction C could be assigned without ambiguity to LVV-hemorphin-7 from both the retention time and matching result.

The retention time of the VVH7-i.r. in fraction A could lead us to suspect two hemorphin-related peptides: hemorphin-7 and VV-hemorphin-6. Nevertheless, several arguments allowed us to attribute the VVH7-i.r. to hemorphin-7. First, better MA and MT values were obtained from spectral comparison with hemorphin-7 standard. Then, it has been already demonstrated that VV-hemorphin-6 was very poorly recognized by IG-C_2 antibodies [17]. Finally, the Picotag amino acid analysis of fraction A revealed the presence of a phenylalanine residue which constitutes the C-terminal amino acid of hemorphin-7 (data not shown). Accurate molecular weights predominant in fractions A, B, C (Fig. 3) deduced from the m/z values of $(M+H)^+$ by substraction of one mass unit for the attached proton were 996.6 (A), 1196.6 (B) and 1308.9 (C). This corroborated absolutely the results deduced from RIA and amino acid analyses and confirmed the generation of hemorphin-7 (fraction A), VV-hemorphin-7 (fraction B) and LVV-hemorphin-7 (fraction C).

4. Discussion

There is growing evidence that altered protease activities are involved in many diseases and could potentially generate a large number of peptides and small proteins. As an example, among this class of molecules, hemoglobin has been identified as a substrate that displays increased proteolysis in Alzheimer's disease cerebellum [5]. The potential for the generation of biologically active peptides from hemoglobin may have important implications for the etiology of Alzheimer's disease. It could provide at least one link between the disease and observed aberrant proteolysis that may be a significant cause of cellular insult and neuronal degeneration [7]. Moreover, some studies report increased levels and activities of cathepsins D, E and B in the aged brain [25] and it is now believed that lysosomal alterations, which accompany various pathologies such as Alzheimer's disease, could led to the accumulation of peptides. Such changes in the neuronal distribution of these lysosomal hydrolases correlated with the presence of hemoglobin in neurons [26] could be related to an increased hemoglobin degradation leading to the release of bioactive peptides.

Now, many authors underline the importance of the enzyme characterization which could be implicated in the in vivo release of bioactive peptides derived from hemoglobin.

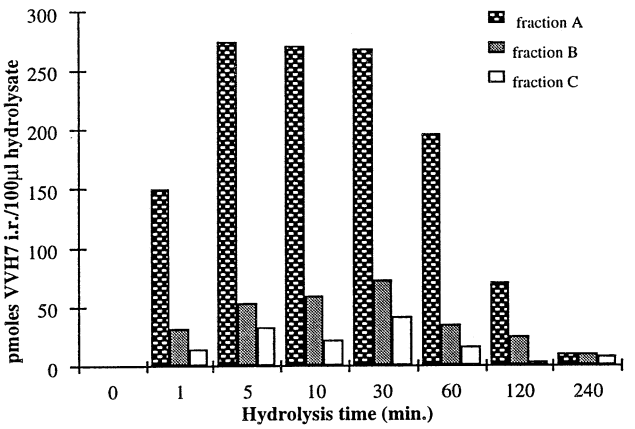

Fig. 2. Kinetics of generation of VV-hemorphin-7 immunoreactive peptides during hemoglobin incubation with lysosomal enzymes.

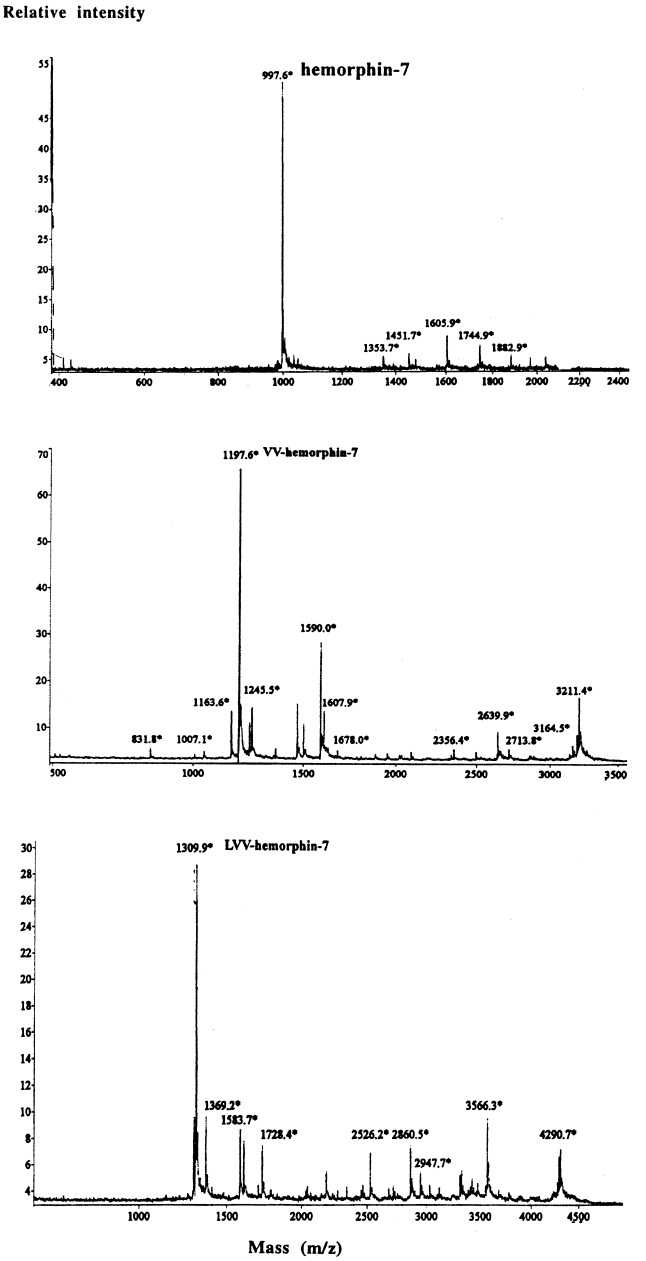

Fig. 3. MALDI mass spectrometry of fractions A, B and C. Positive ions were obtained by MALDI mass spectrometry of fractions A, B, C. The abundant ions at 997.6 (A), 1197.6 (B) and 1309.9 (C) indicate that A, B, C have predominant molecular weights of 996.6 (hemorphin-7), 1196.6 (VV-hemorphin-7), 1308.9 (LVV-hemorphin-7) respectively.

Taking into account these considerations, we used purified lysosomes in order to examine whether acid hydrolases could release hemorphins from hemoglobin. By a discontinuous metrizamide gradient separation, we have been able to isolate a rat liver lysosomal fraction, free of mitochondrial contaminants. Interestingly, an early generation of three hemorphin-7-related immunoreactive peptides (hemorphin-7, LVV-hemorphin-7 and VV-hemorphin-7), mainly represented by hemorphin-7, was observed during co-incubation of hemoglobin with the entire class of lysosomal proteases. Their preference for a primitive cleavage of hemoglobin at this 'strategic zone' [11] may have important implications for the pathobiology of some diseases. From these findings, it may be expected that hemorphins or related peptides might prove to be useful biological markers of increased levels and activities of proteolytic enzymes such as cathepsins.

In a previous study, we observed that the co-incubation of globin with macrophages during 24 h led to the generation of only one hemorphin: VV-hemorphin-7 [12]. The weak sensitivity of the spectral scan analysis used in this previous study could explain the detection of only one hemorphin. It was then also suggested that the protease implicated in the degradation of globin into hemorphin was a cathepsin D-like enzyme. This hypothesis was reinforced by the demonstration that this peptide was generated from hemoglobin by purified cathepsin D and exhibited a good stability towards this enzyme [13,17]. Moreover, when hemoglobin was incubated with cathepsin D, no hemorphin-7 was detected neither by UV spectral analysis nor by RIA [17]. In the present work, we have followed the generation of three hemorphins. Their levels reached a plateau at 5 min and, in contrast to the other studies [12,13,17], they decreased after 1 h. After 2 h no peptides could be detected by UV and only few pmoles of VVH7-i.r. were detectable.

Incubation of globin with macrophages [12] and hemoglobin with purified cathepsin D [13,17] when conducted in the optimal conditions for cathepsin D activity (pH 3.5, 37°C), could prevent other proteolytic activities like aminopeptidase and carboxypeptidase, to be fully expressed. Experimental conditions used in the present study closely resembles the in vivo ones (pH 4.5, 37°C) as reported by Bohley and Seglen [27]. At such a pH, the battery of proteases classically involved in protein degradation were able to exert their proteolytic activities and might act synergistically to ensure hemoglobin hydrolysis. At pH 4.5, both aspartic proteases cathepsins D and L are fully active and may be responsible for the early release of VV- and LVV-hemorphin-7. The presence of hemorphin-7 as soon as 1 min of hydrolysis could be the result of the downstream action of an exopeptidase like cathepsin C, which is present in mammalian lysosomes [28]. In fact, this enzyme is reported to act as a diaminopeptidase at a pH comprised between 4 and 6, and to exhibit significant preference for Phe, Leu or Tyr residues at the P1 site and for Gly or Pro at the P1′ cleavage site [29]. In this study, we observed that neither VV-hemorphin-7 nor hemorphin-7 constituted the final product of hemoglobin processing. Nevertheless, the half-life of hemorphin-7 seems to be long enough to exert an activity for example in the inflammatory response [30]. A carboxypeptidase activity could be expected since a slow degradation of VVH7-i.r. was observed after 1 h of incubation. The proteolytic pathway involved in hemorphin generation from hemoglobin by lysosomal enzymes seems to be the consequence of an ordered process: the polypeptides released as a result of an early digestion by cathepsin D provide substrate for exoproteases like cathepsin C and carboxypeptidase. Studies using specific enzyme inhibitors should be useful to explore this proteolytic pathway.

Finally, as a result of that process a large number of peptides is formed from hemoglobin, which accompanies either a pathology or a physiological state related with an outburst of protease activity. The components of that 'tissue-specific peptide pool' could constitute a part of a general system of peptidergic regulation of tissue homeostasis [2]. However, additional studies both in laboratories and in the clinics will be necessary to appraise with accuracy the role of hemorphins in physiology or pathology.

Acknowledgements: We thank The Conseil Régional Poitou-Charentes for financial support (I.F. Ph.D. grant).

References

[1] Brantl, V., Gramsch, Ch., Lottspeich, F., Mertz, R., Jaeger, K.H. and Herz, A. (1986) Eur. J. Pharmacol. 125, 309–310.
[2] Ivanov, V.T., Karelin, A., Philippova, M.M., Nazimov, I.V. and Pletnev, V.Z. (1997) Biopolymers 43, 171–188.
[3] Fukui, K., Shiomi, H., Takagi, H., Hayashi, K., Kiso, Y. and Kitagawa, K. (1983) Neuropharmacology 22, 191–196.
[4] Zhu, Y.X., Hsi, K.L., Chen, Z.G., Zhang, H.L., Wu, S.X., Zhang, Z.Y., Fang, F.P., Guo, S.Y., Kao, Y.S. and Tsou, K. (1986) FEBS Lett. 208, 253–257.
[5] Slemmon, J.R., Hughes, C.M., Cambell, G.A. and Flood, D.G. (1994) J. Neurosci. 14, 2225–2235.
[6] Pivnik, A.V., Rasstrigin, N.A., Philippova, M.M., Karelin, A.A. and Ivanov, V.T. (1996) Leuk. lymphoma 22, 345–349.
[7] Slemmon, J.R., Wengenack, T.M. and Flood, D.G. (1997) Biopolymers 43, (2) 157–170.
[8] Glämsta, E.-L., Mörkrid, L., Lantz, I. and Nyberg, F. (1993) Regul. Peptides 49, 9–18.
[9] Nyberg, F., Sanderson, K. and Glämsta, E.L. (1997) Biopolymers 43, 147–156.
[10] Carraway, R.E., Mika, S.P. and Cochrane, D.E. (1987) J. Biol. Chem. 262, 5968–5973.
[11] Piot, J.M., Zhao, Q., Guillochon, D., Ricart, G. and Thomas, D. (1992) Biochem. Biophys. Res. Commun. 189, 101–110.
[12] Dagouassat, N., Garreau, I., Sannier, F., Zhao, Q. and Piot, J.M. (1996) FEBS Lett. 382, 37–42.
[13] Fruitier, I., Garreau, I. and Piot, J.M. (1998) Biochem. Biophys. Res. Commun. 246, 719–724.
[14] Zhao, Q., Sannier, F. and Piot, J.M. (1995) J. Liq. Chromatogr. 18, (1) 93–103.
[15] Zhao, Q., Sannier, F., Garreau, I., Lecœur, C. and Piot, J.M. (1996) J. Chromatogr. A 723, 35–41.
[16] Zhao, Q. and Piot, J.M. (1998) Peptides 19, (4) 759–766.
[17] Garreau, I., Cucumel, K., Dagouassat, N., Zhao, Q., Cupo, A. and Piot, J.M. (1997) Peptides 18, 293–300.
[18] Hunter, W.M. and Greenwood, F.C. (1962) Nature 194, 495–496.
[19] Wattiaux, R., Wattiaux-De-Coninck, S., Ronveaux-Dupal, M.F. and Dubois, F. (1978) J. Cell Biol. 78, 349–368.
[20] Aniento, F., Roche, E., Cuervo, A.M. and Knecht, E. (1993) J. Biol. Chem. 268, (14) 10463–10470.
[21] Tsou, K.C. (1956) J. Am. Chem. Soc. 78, 6139–6144.
[22] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[23] Bidlingmeyer, B.A., Cohen, S.A., Tarvin, T.L., Napier, J.R. and Hancock, W.S. (1984) J. Chromatogr. 336, 93–104.
[24] Glämsta, E.L., Marclund, A., Hellman, U., Wernstedt, C., Terenius, L. and Nyberg, F. (1991) Regul. Peptides 34, 169–179.
[25] Schwagerl, A.L., Mohan, P.S., Cataldo, A.M., Vonsattel, J.P., Kowall, N.W. and Nixon, R.A. (1995) J. Neurochem. 64, 443–446.

[26] Ohyagi, Y., Yamada, T. and Goto, I. (1994) Brain Res. 635, 323–327.
[27] Bohley, P. and Seglen, P.O. (1992) Experentia 48, 151–157.
[28] Brindley, P.J., Kalinna, B.H., Dalton, J.P., Day, S.R., Wong, J.Y.M., Smythe, M.L. and McManus, D.P. (1997) Mol. Biochem. Parasitol. 89, 1–9.
[29] Beynon, R.J. and Bond, J.S. (1989) in: D. Rickwood and B.D. Hames (Eds.), Proteolytic Enzymes, a Practical Approach, Practical Approach Series, IRL Press, Oxford, England.
[30] Sanderson, K., Nyberg, F. and Khalil, Z. (1998) Inflamm. Res. 47, 49–55.