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Generation of VV-hemorphin-7 from globin by peritoneal macrophages

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Abstract Bovine globin has been incubated with mice peritoneal macrophages in order to study its hydrolysis by lysosomal enzymes, among which chiefly cathepsin D. Analysis of resulting peptides, by reversed-phase high-performance liquid chromatography (RP-HPLC), shown the release of a bioactive peptide, VV-hemorphin-7. When a carboxyl proteinase inhibitor such as pepstatin A was added, no hemorphin was generated. Our results clearly demonstrated that VV-hemorphin-7 generation was principally due to cathepsin D. This study allowed us to hypothetize a possible pathway for in vivo hemorphins appearance from globin catabolism by macrophages.

Key words: Hemorphin; Hemoglobin; Macrophages; Lysosomal enzymes; Cathepsin D; High-performance liquid chromatography

1. Introduction

A few years ago, biologically active peptides with affinity for opioid receptors were isolated and purified in vitro, from enzymatically treated hemoglobin and in vivo in biological fluids. Because of their origin (hemoglobin), these peptides have been called hemorphins.

The first two hemoglobin-derived peptides identified in vitro and termed hemorphin-4 (Tyr-Pro-Trp-Thr) and hemorphin-5 (Tyr-Pro-Trp-Thr-Gln) were obtained by treatment of bovine blood with gastrointestinal enzymes. These amino acid sequences corresponded to the 34–37 and 34–38 fragments of the beta-chain of bovine hemoglobin and 35–38 and 35–39 fragments of the beta-chain of human hemoglobin [1]. More recently, two additional opioid peptides corresponding to the sequence 32–40, Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe (VV-hemorphin-7) and 31–40, Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe (LVV-hemorphin-7) of the beta-chain of bovine hemoglobin have also been isolated in vitro from a bovine hemoglobin peptic hydrolysate [2].

Concurrently, some similar active peptides have been identified in the organism. Indeed, LVV-hemorphin-4, designated spinorphin (Leu-Val-Val-Tyr-Pro-Trp-Thr), has been isolated from bovine spinal cord by Nishimura and Hazaro [3]. In their report, an inhibitory activity of spinorphin toward some enkephalin-degrading enzymes was demonstrated, suggesting that this substance should be an in vivo neuromodulator. During the extraction and purification of a tetrapeptide, the Tyr-Mif-1 from bovine hypothalamic tissue, another peptide was identified as Val-Val-Tyr-Pro-Trp-Thr-Gln showing homology with residues 32–38 of the beta-chain of bovine hemoglobin. This peptide was called valorphin [4]. Another opioid peptide (LVV-hemorphin-6) was isolated from fresh frozen human pituitaries by Glämsta et al. [5]. Its primary structure (Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg) was identical to fragment 32–40 of the beta-chain of human hemoglobin. The same authors described the presence of LVV-hemorphin-7 in cerebrospinal fluid (CSF) of patients with cerebrovascular bleeding [6], and hemorphin-7 in human plasma after long distance running [7,8]. Many other fragments of the beta-chain of hemoglobin have also been identified in vivo [9].

Concerning the biological properties of hemorphins, their opioid activity was demonstrated by the use of electrically stimulated myenteric plexus/longitudinal muscle preparation of the guinea-pig ileum (GPI) [10]. In this bioassay, hemorphins led to the inhibition of electrically induced GPI contractions. This inhibition was reversed by the specific opioid antagonist, naloxone [11,12]. In addition to this effect, the hemorphins exhibit inhibitory action on angiotensin converting enzyme [13,14]. Another interesting feature of some hemorphins is their effect on β-endorphin release from the pituitary [15]. Moreover, Davis et al. reported that hemorphin-4 and -5 could exert in vivo effect on central nervous system: antinociception, inhibition of gastrointestinal propulsion or inhibition of the volume-induced spontaneous micturition reflex [16].

It can be noticed that the above-mentioned hemorphins, whatever their source, originated from the same region of the beta-chain of hemoglobin (residues 32–41 of human and 31–40 of bovine hemoglobins) [2,17]. These data suggested that hemoglobin could be a precursor of biologically active peptides, and that hemorphins could be generated in the organism during physiological (catabolism of red cells) or physiopathological (inflammation) hemoglobin proteolysis [2,6,17]. In any case, the actual site of hemorphins generation in the organism is still not elucidated.

In the present work, we were interested to determine the possible way of natural generation of hemorphins in the organism. According to the fundamental knowledge of the physiology of macrophages and their role in the cellular catabolism and/or in the inflammatory process, we have investigated the appearance of hemorphins when hemoglobin is submitted to the action of macrophage acidic lysosomal proteases. Thus, bovine globin was incubated with mice peritoneal macrophages. The hydrolysis was followed using UV-spectrophotometry, and high-performance liquid chromatography (HPLC) coupled with photodiode array detection was performed in order to detect the hemorphins generated during the incubation [18].

2. Materials and methods

2.1. Materials and chemicals

UV-absorbance was monitored with a Perkin Elmer lambda 16 spectrophotometer. 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 detec-

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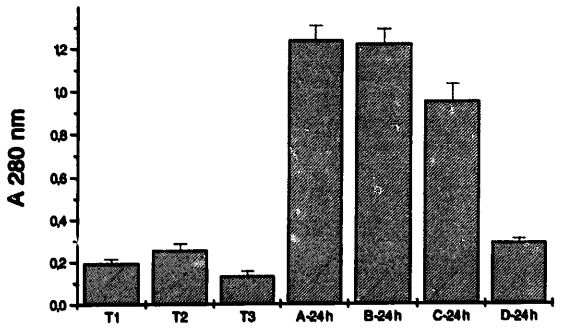

Fig. 1. Proteolytic degradation of globin by macrophages after 24 h incubation and the effects of acidic protease inhibitors, leupeptin and pepstatin A. Globin (10 mg/ml, citrate buffer pH 3.5) was incubated with macrophages monolayer in the absence of glucose (A–24 h), in the presence of glucose 1 g/l (B–24 h), in the presence of glucose 1 g/l and leupeptin 50 μM (C–24 h), in the presence of glucose 1 g/l and pepstatin A (D–24 h). After incubation, the absorbance of the trichloroacetic acid soluble fractions was measured. T1, T2 and T3, control experiments: cells alone, cells with globin without incubation, and globin alone respectively. Mean ± S.E.M., $n = 4$.

tor (Milford, USA). The Millenium software was used to plot, acquire and treat spectral and chromatographic data. A comparison of the UV-spectra (200 to 350 nm) of each peak in the chromatographic profiles with standard hemorphin-7, VV- and LVV-hemorphin-7 spectra (library spectra) was performed during analyses. Standards and samples were chromatographed at room temperature on a Nova-Pak C-18 column. Spectrum matching results were reported by Waters Millenium as Match Angle (MA) and Match Threshold (MT). MA is a measure of the difference in spectral shapes between an unknown spectrum and a library spectrum. MA can range from 0 to 90 degrees. It was found that at lower MA, the spectra were similar, as previously reported [18]. MT described the sensibility of the measure. It can range from 0 to 180 degrees. Larger values indicate a lower sensibility of the measure. If the MA is greater than the MT, the two spectra are different [18].

Amino acids were analysed using a Waters Picotag work station.

Bovine globin, leupeptin, pepstatin A were obtained from Sigma Chemicals Company (Lyon, France). Resazurin Thioglycollate was purchased from Sanofi Diagnostics Pasteur (Marnes la Coquette, France). MEM-E (Minimum Essential Media Eagle), L-glutamine 200 mM, antibiotics (10 000 IU/ml penicillin, 10 mg/ml streptomycin) and fetal calf serum were provided by ATGC Biotechnologie (Paris, France). Hemorphin-7, VV- and LVV-hemorphin-7 were synthesized by C. Guillon, Laboratoire de Technologie Enzymatique, University of Compiègne (Compiègne, France).

All other chemicals and solvents were of analytical grade from commercial sources.

2.2. Macrophage culture

Macrophages were isolated from the peritoneal cavity of female DBF1 mice (25–30 g) Specific Pathogen Free (SPF) following a sterile peritonitis. For this, 1.5 ml of Resazurin Thioglycollate was injected intraperitoneally into each animal. A sterile inflammatory agent such as thioglycollate broth elicits an influx of 10-fold more macrophages into the peritoneal cavity, starting within 1 day and reaching a peak after 3–5 days [19]. In addition, the contents and the specific activities of lysosomal hydrolases were higher in cells from thioglycollate-treated mice than in those from untreated mice [20]. After 4 days, the mice were killed by cervical dislocation. The peritoneal cavity was washed with 5 ml 0.2 M phosphate-buffered saline pH 7.4 (PBS). The peritoneal cells obtained in a single experiment (5 mice) were collected by centrifugation at 400×g for 10 min at 4°C. Macrophages, identified by measuring neutral red uptake, were counted under phase contrast microscopy. The predominant cell type in such peritoneal exudates is the macrophage cell [21]. About 10^7 cells per mouse were obtained and 80% were macrophages.

Purification of macrophages was based on their ability to adherate on culture support. After centrifugation, the cell pellet was resuspended in PBS containing both Mg^{2+} and Ca^{2+} and the cells were incubated in 6-well plates (Falcon) at a density of 4×10^6 macrophages/well in 2 ml of PBS. The incubation was performed at 37°C for 3 h in a humidified atmosphere of 5% CO_2 and 95% air. Ca^{2+} and Mg^{2+} were used as adherence-promoting factors [22]. Non-adherent cells were removed by washing and macrophages were then incubated for 4 days in 2 ml of MEM-E containing 10% fetal calf serum, 20 μM L-glutamine and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin). After 4 days, the macrophages were used in experiments.

2.3. Globin hydrolysis

Globin hydrolysis was performed in 0.1 M sodium citrate buffer pH 3.5 according to Carraway et al. [23].

The ability of macrophages to hydrolyse globin was determined as follows. Bovine globin was diluted in citrate buffer pH 3.5 (10 mg globin/ml) containing 100 UI/ml penicillin and 100 μg/ml streptomycin (Solution A). In order to investigate its putative effect, 1 mg/ml glucose was added to solution A (Solution B). In some experiments, inhibitors of the lysosomal proteases were added in order to characterize which lysosomal proteases should be implicated in globin hydrolysis. Leupeptin (Solution C) or pepstatin A (Solution D) were assayed at a final concentration of 50 μM. The adherent cells were washed twice before use. Macrophage monolayers were incubated with 1 ml of each solution (A to D) at 37°C in humidified atmosphere of 5% CO_2 during 24 h. The incubation was stopped by adding 2 ml of a 12% trichloroacetic acid (TCA) solution. The lysate was then centrifuged at 5000 rpm for 15 min and the pellet was removed. The absorbance of supernatants was measured at 280 nm. Three controls were carried out: cells alone (T1), cells with globin without incubation (T2) and globin alone (T3).

2.4. Reversed-phase high-performance liquid chromatography (RP-HPLC)

The supernatants were resolved on a Waters Nova-Pak silica gel C-18 column (3.9×150 mm). The mobile phase comprised 10 mM ammonium acetate buffer pH 6.0 as eluent A and acetonitrile as eluent B. The flow rate was 1.5 ml/min. The pH of each TCA-supernatant was adjusted to 6.0 with 1 M NH_4OH and filtered through 0.22 μm filters. Then, 30 μl of each sample was injected. On the other hand, synthetic VV-hemorphin-7 (0.5 mg in 30 μl) was co-injected in the same conditions with each supernatant sample. The gradient applied was 0% to 30% of eluent B in 40 min. Between each run, the column was equilibrated with 100% of eluent A for 10 min. UV-absorbance was monitored at 215 nm and 280 nm. Hemorphin-7, VV- and LVV-hemorphin-7 were searched in each eluted peak using the UV-spectra comparison method developed by Zhao et al. [18]. Each hemorphin-related peptide was collected and freeze-dried before amino acid analysis.

2.5. Amino acid analysis

Peptide hydrolysis was achieved with constant-boiling 6 M HCl containing 1% phenol, for 24 h at 109°C. Precolumn derivatization of amino acids with phenyl isothiocyanate and HPLC separation of derivatized amino acids on a Waters RP-Picotag column (3.9×150 mm) were performed according to Bidlingmeyer et al. [24]. The detection wavelength was 254 nm and the flow rate 1 ml/min. All aqueous HPLC eluants were filtered prior to use on Millipore system filtration with 0.22 μm filters and degassed with helium during analysis.

2.6. Mass spectrometry analysis

Mass spectra, generated from FAB mass spectrometry of the active peptide, were recorded on a four-sector "Concept II" tandem mass spectrometer (Kratos, Manchester, UK). Ions were produced in a standard FAB source by bombarding the sample with xenon atoms

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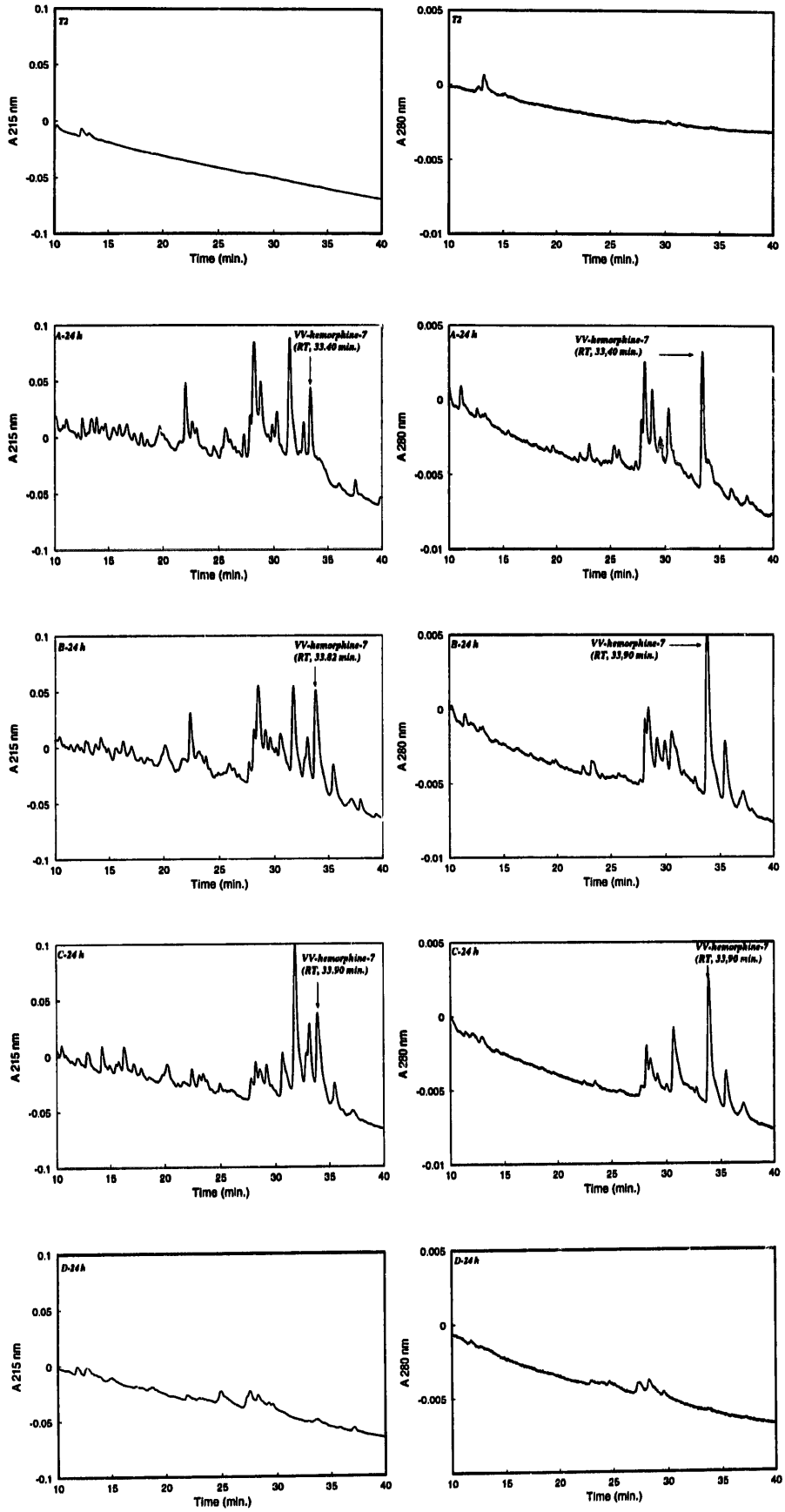
Fig. 2. Reverse phase HPLC of TCA soluble fractions from samples A–24 h, B–24 h, C–24 h, D–24 h and T2 control experiment. UV-absorbance was monitored at 215 nm and 280 nm. Hemorphins were localised by UV-spectra comparison after separation of TCA-supernatants on a Nova-Pak C-18 column. TCA-supernatants were filtered through 0.22 μm filters and 30 μl were applied to the column. The gradient was 0% to 30% of eluent B in 40 min at a flow rate of 1.5 ml/min. An aliquot of hemorphin-like peaks was collected for the amino acid composition analysis. The same profile as T2 was obtained for T1 and T3 control experiments.


T2
A 215 nm
A 280 nm
Time (min.)
A-24 h
VV-hemorphine-7
(RT, 33.40 min.)
B-24 h
VV-hemorphine-7
(RT, 33.82 min.)
VV-hemorphine-7
(RT, 33,90 min.)
C-24 h
D-24 h

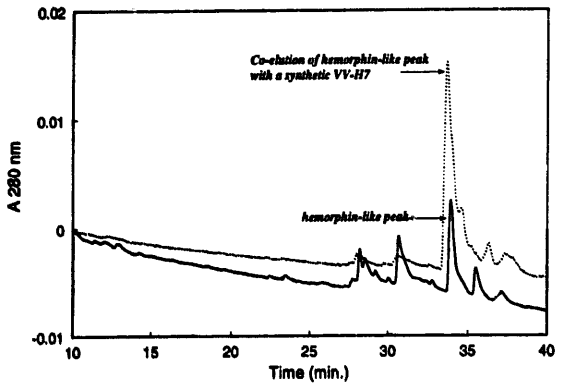


Fig. 3. HPLC co-elution profile of synthetic VV-hemorphin-7 (VV-H7) with hemorphin-like peak from sample C–24 h. Chromatographic conditions were the same as in Fig. 2.

having a kinetic energy of 8 KeV and the instrument was operated at an accelerating voltage of 8 KV. The peptide was dissolved in water (250 μg in 50 μl) and 1 μl of the solution was loaded on the stainless steel tip with thioglycerol as matrix. The mass range was scanned at 10 s/decade with a mass resolution of 3000. Caesium iodide was the standard for mass calibration.

3. Results and discussion

The absorbances at 280 nm of TCA-supernatants from samples incubated 24 h in the absence or presence of inhibitors, are displayed in Fig. 1. These results clearly showed a proteolytic activity due to macrophage cells. After incubation of macrophages with globin and without inhibitor, a strong increase of the absorbance indicated the presence of a large amount of peptides in the supernatants (A–24 h, B–24 h). No significant difference was noticed with (A–24 h) or without (B–24 h) glucose in the incubation media. In the absence of macrophage cells (T3), a very low absorbance was observed after 24 h incubation showing that the cells were crucial for the proteolytic activity. Moreover, in the absence of bovine globin (T1), a very low absorbance was also obtained suggesting the lack of hydrolysis of any other proteinic substrate. Experiments carried out in the presence of enzyme inhibitors were both characterized by a decrease of absorbance at 280 nm. However, in the presence of leupeptin (C–24 h), the decrease was less significant than with pepstatin A (D–24 h). The presence of leupeptin induced about 28% inhibition of the proteolytic activity (C–24 h). As far as pepstatin A is concerned, a complete inhibition was observed since the absorbance was reduced by 100% (D–24 h). The present observations, for both inhibitors, are in agreement with an earlier report about the proteolytic activity of leukocyte(s) proteases and cathepsin D [25]. It was demonstrated that a biologically active xenopsin-related peptide was generated from liver extracts by these enzymes. Pepstatin A has been used as a potent inhibitor of acidic proteases and was shown to be a strong inhibitor of the pepsin-like acid protease, cathepsin D. In addition, leupeptin has also been demonstrated to inhibit cathepsin D activity but to a lesser extent [25]. Our results allowed us to speculate about the ability of macrophage acidic proteases, and chiefly cathepsin D, to hydrolyse globin.

The UV-profiles obtained from fractionation of TCA-supernatants on a C-18 Nova-Pak column are shown in Fig. 2. The resolution of each sample was achieved in less than 40 min. The efficiency of this one-step separation allowed easy on-line detection of hemorphins using the UV-spectra comparison method recently developped by Zhao et al. [18]. This method allowed the detection of an hemorphin-like peak among many other peaks in samples A–24 h, B–24 h, and C–24 h. Moreover, the MA and MT values obtained by UV-spectra comparison suggested that this peptide should be VV-hemorphin-7 (Table 1). This result was verified by the co-elution of this hemorphin-like peak with synthetic VV-hemorphin-7 at a retention time of approximatively 34 min (Fig. 3). This result was confirmed by Picotag amino acid analysis (Table 2). With regard to amino acid composition, the weak hydrolysis of the Val–Val peptide bond accounted for the 1.12 valine residues found per molecule of VV-hemorphin-7. Fig. 4 shows the positive FAB-MS spectrum of this hemorphin-like peak. Accurate relative molecular weight, deduced from the m/z value of $(M+H)^+$ by subtraction of one mass unit for the attached proton is 1194. According to these results and taking into account the known amino acid sequence of α and β bovine globins, it was clear that the composition found was only compatible with peptide of sequence originating at Val residue 32 and extending to Phe-40 in the β-chain. In consequence, the amino acid composition determined for each hemorphin-like peak was in a good agreement with the spectra-comparison analysis and proved unam-

Table 1
Spectrum matching results obtained from the UV-spectra comparison

	RT*	MA	MT	Name**
A–24 h	33.877	0.629	1.238	VV-hemorphin-7
B–24 h	33.838	0.593	1.376	VV-hemorphin-7
C–24 h	33.867	0.723	1.133	VV-hemorphin-7

During chromatographic analysis on a Nova-Pak C-18 column of TCA supernatants, the Match Angle (MA) and Match Threshold (MT) were calculated by Millenium system from the comparison of UV-spectra of synthetic hemorphins with each eluted peak.
*Retention time (min).
**Given by Millenium software to the peak after UV-comparison.

Table 2
Amino acid composition of hemorphin-like peak isolated from B–24 h

Amino acid	Residues/molecule
Asp	—
Glu	0.94
Ser	—
Gly	—
His	—
Arg	0.96
Thr	1.40
Ala	—
Pro	1.17
Tyr	1.00
Val	1.12
Met	—
Cys	—
Ile	—
Leu	—
Phe	1.07
Lys	—
Trp	ND

Amino acid composition analysis was similar for all samples (A–24 h, B–24 h and C–24 h). Results are expressed in amino acid residues per molecule.
ND: not determined.

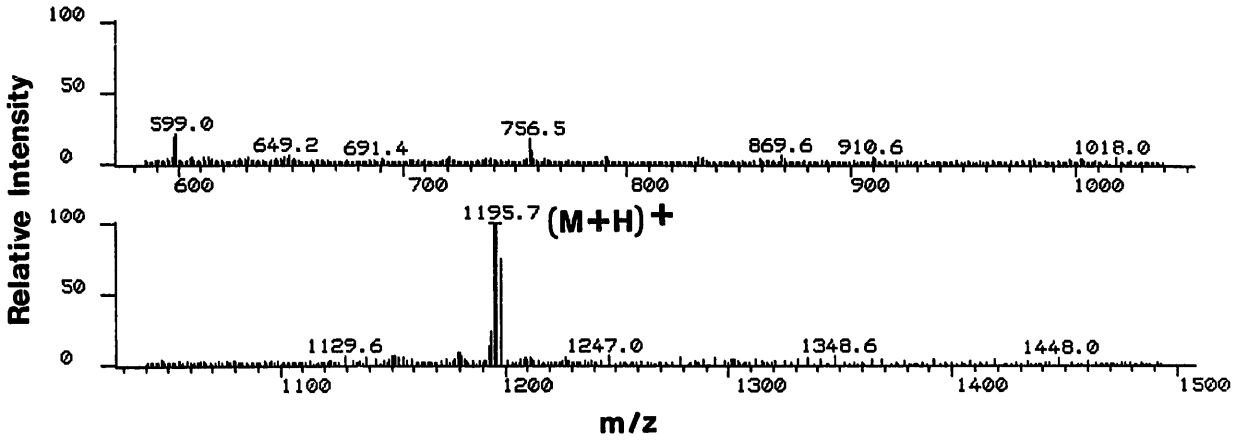


Fig. 4. FAB mass spectrometry of hemorphin-like peak. Positive ions were obtained by FAB mass spectrometry of hemorphin-like peak. The abundant ion at 1195, designated as a molecular cation $(M+H)^+$, suggests a molecular weight of 1194.

biguously that VV-hemorphin-7 (Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe) was generated from globin by the macrophage acidic lysosomal enzymes. It can be noticed that no hemorphin-like peak appeared in sample D-24 h (Fig. 2, D-24 h). All the chromatographic profiles were in good agreement with spectrophotometric measurements.

In consequence, we may further suppose that the VV-hemorphin-7 formation was due to the cathepsin D action. More generally, the cathepsin D was probably responsible of the major proteolytic activity toward globin since no peak was detected when pepstatin A was added in the incubation media (D-24 h). Control samples (T1 to T3) were also injected and no peak was detected.

Previous studies have demonstrated the ability of acidic proteases such as pepsin to generate biologically active VV- and LVV-hemorphin-7 from hemoglobin in vitro [2]. Concurrently, some authors have found hemorphins in biological fluids [6]. Nevertheless, the generation of these bioactive peptides has never been described precisely as the result of a specific in vivo proteolytic attack by cellular enzymes. In the present experiment, we investigated the possibility for macrophage acidic proteases, and especially the pepsin-like enzyme cathepsin D, to liberate hemorphins from globin. It must be pointed out that LVV-hemorphin-7 has not been found in the resulting peptide mixture. Recently, we have demonstrated that during the peptic hydrolysis process of globin, LVV-hemorphin-7 disappeared when the level of VV-hemorphin-7 increased. This study showed that LVV-hemorphin-7 was a precursor of VV-hemorphin-7 [26]. The results presented here suggest that the same process should be involved.

In conclusion, this study gives evidence that macrophage enzyme(s) were able to generate at least one hemorphin from globin. The pH of this process and its inhibition by pepstatin A suggest that cathepsin D is the main enzyme involved. These results are in agreement with previous suggestions about the existence of precursor(s) which could be processed to form biologically active peptides in vivo during states of enhanced phagocytosis and/or enzyme release by macrophage cells [25]. The actual pathway by which VV-hemorphin-7-peptide might be generated and involved physiologically is presently not fully elucided. Some authors have already demonstrated the potent role of hemorphins toward endorphins [15,16] and Angiotensin Converting Enzyme (ACE) [13,14]. These last authors suggested that these opioid-like peptides might act both on blood pressure regulation via ACE and on analgesic strength via opioid receptors during physiological and/or pathologic degradation of hemoglobin [13].

Therefore, in the organism there could be an opioid system, represented by the "classical" opioid peptides (enkephalins, endorphins or dynorphins) and by hemorphins. Since several hemorphins have been found in vivo [3,9] and characterized by their biological properties [13,17], it will be of great interest to find out all the metabolic pathways from hemoglobin to hemorphins. Moreover, Carraway et al. suggested the existence of a putative endogenous processing, similar to the renin–angiotensin system, which generates neurotensin- and enkephalin-related peptides in blood circulation [27,28]. The in vivo processing of plasma precursors may involve a pepsin-like activity releasing bioactive peptides from circulating substrates, as does pepsin in vitro [2] or macrophage acidic proteases as was suggested in the present work. Consequently, we could consider that such as system should be implicated in globin proteolysis in vivo.

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