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ISOLATION AND CHARACTERIZATION OF TWO OPIOID PEPTIDES FROM A BOVINE HEMOGLOBIN PEPTIC HYDROLYSATE

[1]Jean-Marie Piot, [1]Qiuyu Zhao, [1]Didier Guillochon, [2]Guy Ricart and [3]Daniel Thomas

[1]Laboratoire de Technologie des Substances Naturelles, BP 179-IUT A Lille I, 59653- VILLENEUVE D'ASCQ Cédex, FRANCE

[2]Laboratoire de Spectrométrie de Masse, Lille I, 59655- VILLENEUVE D'ASCQ Cédex, FRANCE

[3]Laboratoire de Technologie Enzymatique, URA 1442 du C.N.R.S., Université de Technologie de Compiègne, BP 649, 60206- COMPIEGNE Cédex, FRANCE

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SUMMARY: Two opioid peptides were isolated from a bovine hemoglobin hydrolysate, by use of gel permeation (GP) and reverse phase (RP) high performance liquid chromatography (HPLC). Their primary structure and accurate molecular weights, determined by amino acid analysis and fast atom bombardment (FAB) mass spectrometry, were identical to fragments 31-40 (LVV-hemorphin -7) and 32-40 (VV-hemorphin 7) of the ß-chain of bovine hemoglobin. The same fragments occur in human hemoglobin in positions 32-41 and 33-41 of the ß-chain, respectively. The opioid potency of these peptides , exhibited by use of electrically stimulated muscle of isolated guinea-pig ileum (GPI), were significant and comparable with some others previously described. In addition, the location of the two opioid peptides, VV-hemorphin-7 and LVV- hemorphin-7, revealed the existence of a "strategic zone" both in the bovine and human ß-chains of hemoglobin.

Enzymic hydrolysates of food proteins have for many years found applications in the feeding of certain groups of hospitalised patients or for improving the functional properties of these proteins [1]. Additional applications have been investigated within the past few years such as research on biological activities like opioid activity [2]. Materials with opioid activity have been isolated and purified from enzymatic digests and they were shown to contain pure peptides [3]. Evidences for the identity between opioid substances and isolated peptides were obtained from the opioid activity elicited by synthetic peptides with the same sequences [4]. The first and the most studied biologically active peptides were the opioid peptides isolated from casein enzymatic hydrolysates such as casomorphins [3,5,6]. These peptides, because of their exogenous origin, were named exorphins. Other protein sources like wheat gluten hydrolysates exhibited opioid-like activity [2,7].

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A few years ago, peptides with affinity for opioid receptors were isolated from enzymatically treated blood [8] which constitutes an important and under-utilised source of food proteins [1]. These peptides, named hemorphins were found during the purification procedure of cytochrophins, opioid peptides derived from mitochondrial cytochrom b, originally obtained by treatment of bovine blood with a mixture of gastrointestinal enzymes [9]. Two of these hemorphins (Tyr-Pro-Trp-Thr and Tyr-Pro-Trp-Thr-Gln) termed hemorphin-4 and hemorphin-5, respectively, were identified by computer search to the 34-37 and 34-38 fragments of the beta-chain of bovine hemoglobin and the 35-38 and 35-39 fragments of human hemoglobin. Recently, an opioid active fragment of hemoglobin was isolated from the human pituitary gland [10]. This peptide (LVV-hemorphin 6) corresponds to the sequence at position 32-40 of the beta chain of human hemoglobin. All of these hemorphins exhibit an opioid activity determined by use of electrically stimulated myenteric plexus/longitudinal muscle preparation of the guinea-pig ileum (GPI) [9,10]. All induced inhibitions of the GPI contractions by the peptides were reversed or blocked by (-) - naloxone, and hemorphin-6 appeared the most potent form causing a 50% inhibition .

On the basis of these previous observations, we decided to investigate if a peptone preparation, obtained in our laboratory by peptic hydrolysis of bovine hemoglobin, when used in clinics, might have side effects such as those due to production of opioid peptides. In the present work, we have fractionated a peptic bovine hemoglobin digest, obtained at pilot-plant scale by an ultrafiltration process [11]. Protein fractions with highest opioid-like activity, determined by use of electrically stimulated muscle contraction of isolated GPI, were further separated into nearly pure peptides by a combination of GP (gel permeation) and RP (reverse phase) HPLC (high pressure liquid chromatography) and analysed by FAB (fast atom bombardment-mass spectrometry) [12]. The peptides structures were elucidated by amino acid analysis. They were located in the well known bovine globin sequence and compared with previously described hemorphins. Identical synthetic peptides were tested for their opioid activity.

MATERIALS AND METHODS

Materials

All common chemicals and solvents were of analytical grade from commercial sources. Acetonitrile was of HPLC grade. All aqueous HPLC eluents were filtered prior to use on Sartorius (Palaiseau, France) 0.45 µm filters, and degassed with helium during analysis. The synthetic peptides used in this study were synthetized by C.Guillon, Laboratoire de Technologie Enzymatique, University of Compiègne, Compiègne, France. Normorphine hydrochloride , (-)- naloxone and pepsin were purchased from Sigma Chemicals (St. Louis, USA). The guinea-pig ileum contractions were recorded with a Washington isotonic transducer.

Methods

Hydrolysate preparation.Bovine hemoglobin hydrolysate was obtained at pilot-plant scale by peptic proteolysis in an ultrafiltration reactor as previously described in reference

[11].Hemoglobin obtained by hemolysing erythrocytes was added to water and 4 M hydrochloric acid to obtain 300 l of 5 % denatured hemoglobin at pH2. For peptic digestion, 80 l of hemoglobin were heated to 40°C in a reactor and added to porcine pepsin (EC.3.4.23.1.— 240 Anson units). Hydrolysis lasted 8 h and pH2 was maintained by a pH stat. After 8 h, the reactor was connected to ultrafiltration membranes (mineral membranes, with nominal molecular weight cut off lower than 20,000 daltons) with an area of 0.32 m^2. Ultrafiltration was conducted at 40° C for 22 h. Operating pressures were adjusted in order to keep a constant ultrafiltration flow (10 l/h). Incubation volume was maintained constant in the ultrafiltration reactor by adding hemolysate. The ultrafiltrate was desalted by electrodialysis and then atomized. The nitrogen content of the peptidic powder was determined by the Kjeldahl method and allowed us to evaluate amounts of peptides in the hydrolysate greater than 90 % (N x 6.25). The percentage of free amino acids was estimated under 1 %.

GP and RP HPLC. A Waters 600 E system was used for the GP-and RP-HPLC separations.
GP-HPLC. Separations were performed on a 60 cm x 21,5 mm i.d. TSK G2000 SWG column eluting with 10 mM ammonium acetate buffer (prepared daily from analytical ammonium acetate and adjusted to pH 6.0 with acetic acid). Hydrolysate powder samples of 50 mg were dissolved in 500 µl of the same buffer and filtered through 0.2 µm filters before being applied to the column. The flow rate was 6 ml/min. Fractions were collected and freeze dried.
RP-HPLC. The active fraction eluted from TSK G2000 SWG was analysed by RP-HPLC on a 30 cm x 19 mm i.d. Delta Pak C-18 column. The mobile phase comprised 10 mM ammonium acetate buffer pH 6.0 as eluent A and acetonitrile as eluent B. The flow rate was 12 ml/min. Samples were dissolved in buffer A (200 mg/ml) filtered through 0.20 µm filters and 500 µl were injected. The gradient applied was 0-40% B in 40 min. Re-equilibration at 0% B for 10 min was performed between each analysis. Fractions were collected and freeze dried.

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 having a kinetic energy of 8 KeV and the instrument was operated at an accelerating voltage of 8KV. 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.

Amino acid analysis.Amino acids were analysed using a Waters Picotag Work Station. Peptide hydrolysis was achieved with constant-boiling HCl containing 1% phenol, for 24 h. at 100°C. Precolumn derivatization of amino acids with phenyl isothiocyanate and HPLC separation of derivatized amino acids on a waters RP- Picotag column (150 mm x 3.9 mm i.d.) were performed according to Bidlingmeyer et al. [13]. The detection wavelength was 254 nm and the flow rate 1 ml/min.

Determination of opioid activity in guinea pig ileum preparation. All experiments were performed on the guinea-pig isolated ileum according to Kosterlitz et al [14] and Brantl et al [3] . The terminal ileum portion was used after the 10 cm nearest to the ileo-caecal junction had been routinely discarded, and the opioid activity was tested on the contractions of the longitudinal muscle of a segment of ileum. The organ bath contained 5ml Krebs solution at 37°C, pH 7.4, aerated with 95% O_2 and 5% CO_2.The tissue was stimulated with rectangular pulses at a frequency of 0.1 Hz, 1 ms duration and supramaximal voltage (40 V). In this isolated organ preparation, opioid activities were evaluated in terms of an inhibition of electrically induced contractions, in so far as these proved antagonizable by the opiate antagonist (-) - naloxone (0,2 µM). From these effects IC_{50} (concentration causing a 50% inhibition) values were calculated for quantitative evaluation.

RESULTS AND DISCUSSION

The UV-profile from the fractionation of a total peptic hydrolysate of hemoglobin on the TSK column, is shown in Fig. 1. The analysis of 50 mg of this peptic digest exhibited nine peptidic fractions and the resolution was achieved in less than 50 min with a quite similar efficiency to analytical separation [12]. Such separation allows easy collection of any fraction to be tested. One active peptidic fraction (FVII) found among several inactive others, was collected for further separation by RP-HPLC. By this technique, the active fraction was resolved into two peaks (Fig. 2) eluted very next to each other at 34% (AI) and 36% (AII) of acetonitrile respectively. Each peak was subjected to an additional separation on RP-HPLC in order to check its purity (not shown). Based on this data, the two peptidic peaks obtained in significant amounts from the semi-preparative RP-HPLC were used throughout the present work for amino acid analysis, mass spectrometry and opioid activity studies.

Fig. 3 shows the positive FAB-MS spectra of these selected peptides. Accurate relative molecular weights , deduced from the m/z values of $(M + H)^+$ by substraction of one mass unit for the attached proton are 1194 (AI) and 1307 (AII). Amino acid analysis of AI and AII gave results as shown in Table I. This corroborated absolutely the molecular weights previously determined. When the known amino acid sequences of α

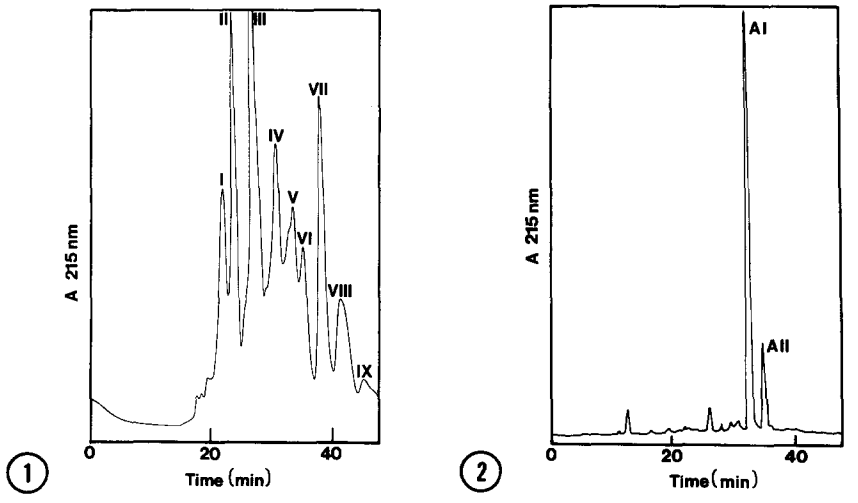

FIG.1. **Gel filtration HPLC of peptic digest of bovine hemoglobin.** The hydrolysis was carried out as described in " Materials and Methods". Total hydrolysate was chromatographed on a TSK G2000 SWG column in 10 mM ammonium acetate buffer (pH 6.0) with a flow rate of 6ml/min. Fractions were tested for opioid activity. One active peptidic fraction (F VII) found among several inactive others was collected for further separation by RP-HPLC.

FIG.2. **Purification of active peptides on a reverse-phase HPLC column.** Peak VII from the TSK G 2000 SWG column was applied to a Delta-Pak C18 column and eluted with a mobile phase consisting of 10 mM ammonium acetate buffer, pH 6.0,as eluent A and acetonitrile as eluent B. The gradient was 0 to 40% B for 40 min with a flow rate of 12ml/min. An aliquot of each peak was collected and assayed for their opioid activity (GPI test). Active peptidic peaks (AI and AII) were then rechromatographed under the same conditions to assess their purity (not shown).

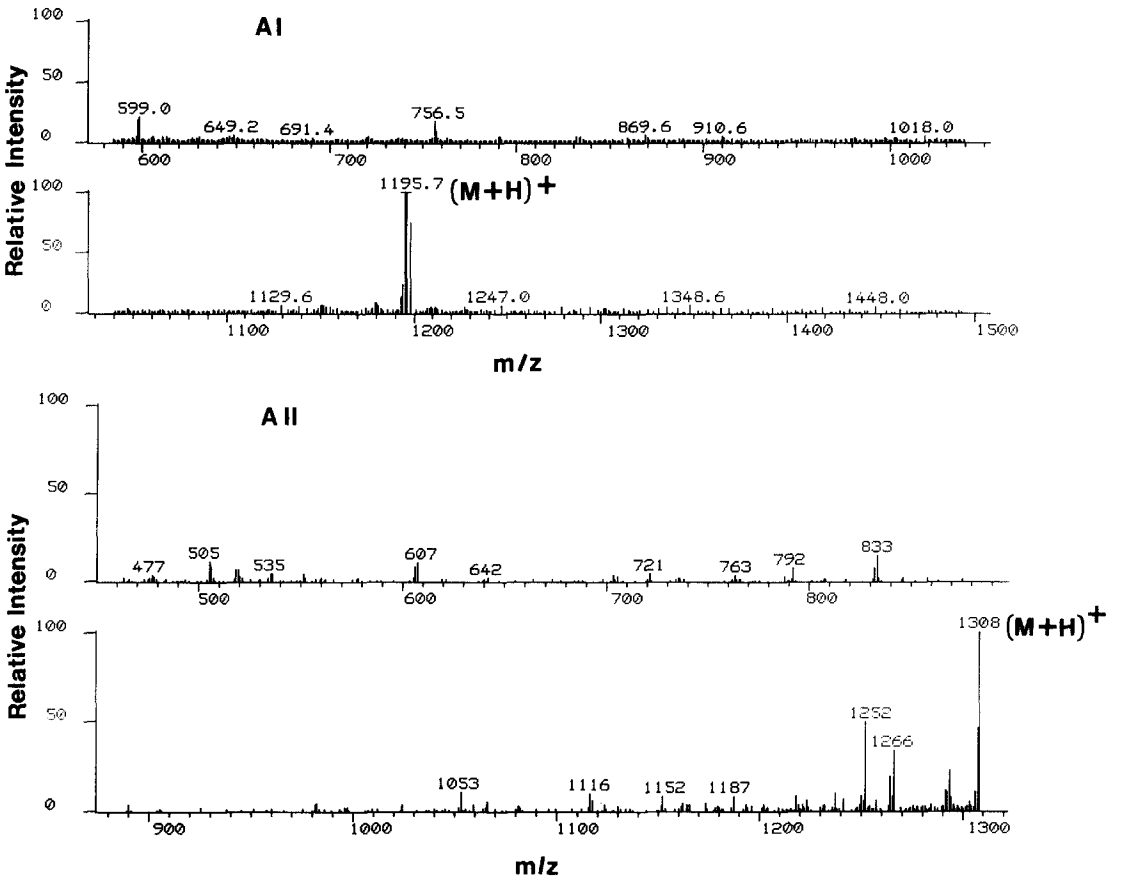

FIG.3. **FAB mass spectrometry of peptides AI and AII.** Positive ions were obtained by FAB mass spectrometry of AI and AII. The abundant ion at 1195, designated as a molecular cation $(M+H)^+$, suggests that AI has a molecular weight of 1194. The abundant ion at 1308 suggests a molecular weight of 1307 for AII.

and β bovine globins were examined for regions containing these amino acids, it was clear that the composition found was only compatible with peptides of sequences originating at Val residue 32 and extending to Phe-40 (AI) and Leu-31 to Phe-40 (AII), both in the β -chain (Fig. 4).

It should be observed that these two peptides, differing only from one another in the presence of Leu-31 (AII), were particularly well separated in RP-HPLC in spite of the substantial quantity of peptidic sample loaded on the column (100 mg versus 2 mg in analytical conditions [12]). This is probably due to the presence of Leu-31 increasing hydrophobicity. The synthetic peptides Val-Val-Tyr-Pro-Trp-Thr- Gln-Arg-Phe (32-40) and Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe (31-40) have identical chromatographic properties as the isolated peptides.

In Fig. 5, a log dose-response relationship in the GPI preparation for the opioid effect of isolated peptides AI and AII is shown. The points of the curves represent the mean of data obtained with three different pieces of muscle. Thus, in the GPI assay, both AI and its N-terminal extension Leu-AI, that is to say AII, were able to inhibit the

TABLE I. **Amino acid composition of opioid peptides AI and AII isolated from peptic hydrolysate of bovine hemoglobin.** Amino acids are expressed in residues per molecules. ND indicates that individual amino acids were not determined.

Amino acid	A I	A II
Asp		
Glu	1.04	1.12
Ser		
Gly		
His		
Arg	1.04	0.96
Thr	0.92	0.91
Ala		
Pro	1.04	1.00
Tyr	1.00	1.00
Val	1.82	2.19
Met		
Cys		
Ile		
Leu		0.97
Phe	1.04	0.92
Lys		
Trp	(ND)	(ND)

electrically induced muscle contraction. Importantly, all induced inhibitions of the GPI contractions by the peptides were reversed by the opiate antagonist (-) - naloxone (0.2 μM). The peptide AI appeared slightly less potent than its extended form.Table II present the opioid activities of these opioid peptides and normorphine in comparison to

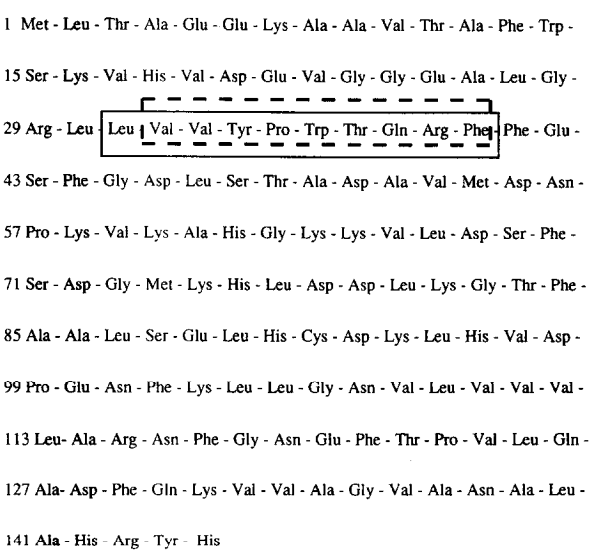

FIG.4. **Amino acid sequence of peptides AI and AII.** The complete sequence of the ß-chain of bovine hemoglobin is presented. Amino acid sequences of AI (---) and AII (—) are boxed.

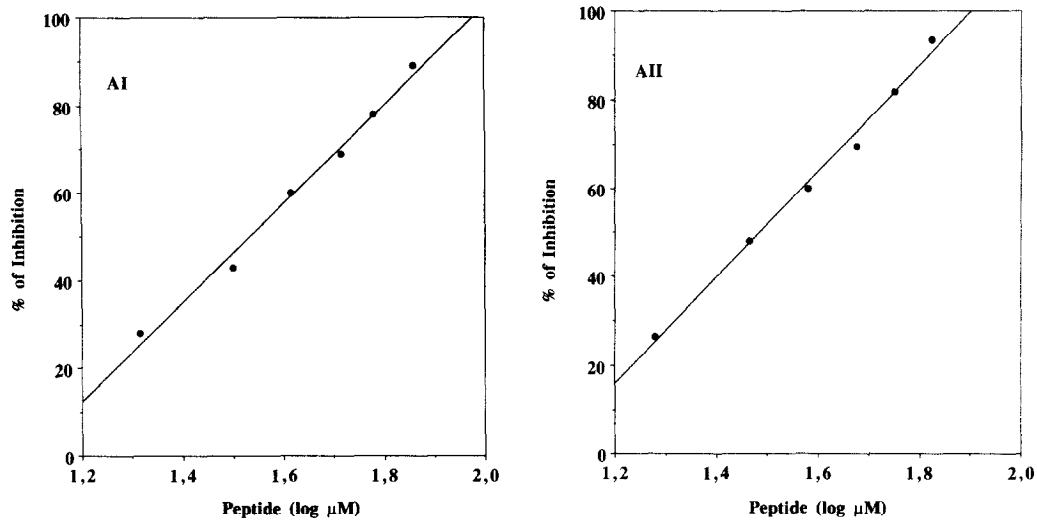

FIG.5. **Opioid activities of peptides AI and AII.** The log-dose response effect of peptides AI and AII, on inhibition of the contractions of electrically stimulated guinea-pig ileum, was determined as decribed under" Materials and Methods". Each data point represents the mean value of three independent experiments. All induced inhibitions were reversed by (-) - naloxone (0.2 μM).

previously described hemorphins-4, -5, -6 and LVV-hemorphin-6 [8,10]. In view of their origin and activity, the new nona- and decapeptide were named VV-hemorphin-7 (AI) and LVV-hemorphin-7 (AII), respectively. Interestingly these hemorphins, as

TABLE II. Opioid activites of LVV-hemorphin-7 and VV-hemorphin-7 in comparison with previously described hemorphins. IC_{50} values indicate substance concentration (μM) causing 50% inhibition of electrically induced contraction of the guinea-pig ileum muscle preparation. Our values(*) are given by means from three separate experiments

Substance	IC_{50} - values (μM)	Ref.
Hemorphin - 4	45.2	8
Hemorphin - 5	46.1	8
Hemorphin - 6	4.3	10
Hemorphin - 7	2.92	*
LVV- Hemorphin - 6	90	10
AII (LVV-Hemorphin-7)	29.1	*
AI (VV-Hemorphin-7)	34.3	*
Normorphin	0.08	*
Normorphin	0.1	8

evaluated by their IC_{50} values, are less potent than hemorphin-6 but more potent than hemorphins-4 and -5 and much more potent than LVV- hemorphin-6. Moreover it is important to note that VV-hemorphin-7 and LVV-hemorphin-7 have a lower opioid activity than most of the β-casomorphins [10,15].The identical synthetic peptides were tested for their opioid potency and exhibited the same activities as the isolated peptides.We have synthesized hemorphin-7 (hemorphin-6 plus the Phe) and tested it in our GPI system (Table II). Thus, it appeared that the C-terminal Phe residue did not significantly increase the potency of this new peptide in comparison with hemorphin-6, as previously described by Glämsta et al [16] .

It is interesting , as mentioned in the introduction to compare our isolated peptides with previously described hemorphins obtained either from the bovine [8]or human [10] β-chain of hemoglobin. It can be noticed that all these hemorphins, whatever their source, originated from the same region of the β- chain. This part of the molecule (residues 32 → 40 of human and residues 31 → 40 of bovine β-globins) seems to play a biological role and could be considered as a "strategic zone" of β-globin (Table III). Such a "strategic zone" was already described for bovine β-casein from which originate many opioid peptides [6]. As far as the release of hemorphins is concerned, Brantl et al [8] used a gastrointestinal enzymes mixture and Glämsta et al [10,16] suggested the action of a trypsin-like and chymotrypsin-like enzyme. In our work, pepsin was used for hydrolysing hemoglobin [11]. Such peptic hydrolysis was previously related by Zioudrou et al [2] for the production of exorphins from food proteins.

Thus, it seems that hemorphins are hidden in an inactive state inside the hemoglobin structure. To exert biological effect in vivo, they must be either produced in the intestinal tract from foods containing blood [17] but resist complete degradation by

TABLE III. Localisation of hemorphins from human and bovine hemoglobins: characterization of a "strategic zone"

	Bovine hemoglobin ß - chain	Human hemoglobin ß - chain
	31 40	32 41
" Strategic zone"	Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe
Hemorphins		
Hemorphin-4	Tyr-Pro-Trp-Thr (8)	Tyr-Pro-Trp-Thr (8)
Hemorphin-5	Tyr-Pro-Trp-Thr-Gln (8)	Tyr-Pro-Trp-Thr-Gln (8)
Hemorphin-6		Tyr-Pro-Trp-Thr-Gln-Arg (10)
LVV-Hemorphin-6		Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg (10)
VV-Hemorphin-7	Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	
LVV-Hemorphin-7	Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	

intestinal proteases, or be formed in the organism during the physiological or pathological degradation process of hemoglobin [8]. It is interesting to speculate that LVV - hemorphin-7 or VV - hemorphin-7, both originally produced by pepsin-like enzymes, could be after that metabolized to more or less active forms such as hemorphins-4, -5, -6. It should be noted that metabolism of peptides into shorter fragments is not uncommon among the opioid family of peptides [18]. Recent studies [19] about the stability of hemorphin-6 shown that when this peptide was incubated with a lung enzyme extract, the Thr - Gln bond was cleaved and the hemorphin-4 fragment was formed. As far as the transformation of LVV-hemorphin-7 into LVV-hemorphin-6 is concerned, the latter could be possibly obtained by the action of a trypsin-like enzyme (hydrolysis of Arg39 - Phe40).Since previously reported by Brantl et al.[8], opoid active fragments may be produced *in vitro* from bovine hemoglobin, further investigations need to be done in this area so as to determine the kinetic of release of hemorphins resulting from hemoglobin hydrolysis by pepsin.This could by another way, help us to establish partially the very intricate kinetic model for hydrolysis of this fairly simple and well-defined substrate. In order to get a better knowledge of this mechanism, FAB and tandem mass spectrometry coupled with HPLC seem to be the most appropriate method as lately described for the analysis of bovine β-casein tryptic digests [20] , such studies are now undertaken in our laboratory.

Moreover, it would be particularly interesting to determine the receptor activity of these peptides, as they do not have any known structural homology to other opioid peptides [21]. One may reasonably suppose that LVV- hemorphin-7 and VV-hemorphin-7 could exert a binding affinity for μ opioid receptors, in regards to the results previously obtained with LVV- hemorphin-6 [10], and this in spite of the absence of a free amino terminal tyrosine which is considered important in opioid peptide - receptor interactions [18, 22].

In the present study the formation of two opioid peptides, LVV- and VV-hemorphin-7, from peptic hydrolysis of hemoglobin, has been proved. It would be a real challenge to find if these blood-borne peptides may occur as degradation products in vivo and then can exert a physiological activity, although they exhibit rather unclassical opioid structures.

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