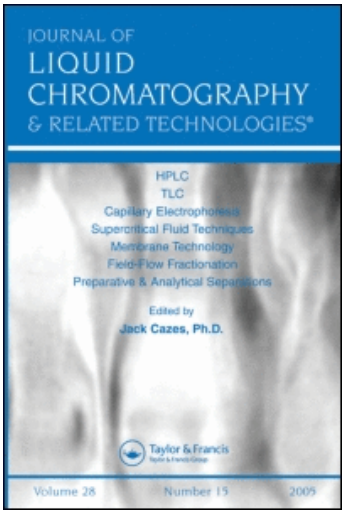

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A Rapid Detection and Identification of Hemorphins Released from Bovine Hemoglobin Enzymatic Hydrolysis by Use of HPLC Coupled with Photodiode Array Detector

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A RAPID DETECTION AND IDENTIFICATION OF HEMORPHINS RELEASED FROM BOVINE HEMOGLOBIN ENZYMATIC HYDROLYSIS BY USE OF HPLC COUPLED WITH PHOTODIODE ARRAY DETECTOR

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ABSTRACT

Identification of hemorphins issued from a complex hemoglobin enzymatic hydrolysate was carried out by UV-spectra comparison. Two hemorphins,VV-hemorphin-7 and LVV-hemorphin-7, were detected in a single step by the use of HPLC coupled with photodiode array detector. This technique greatly simplified the the multistage identification and purification strategy. This method could also be efficiently applied to the identification of peptides containing aromatic amino acids.

INTRODUCTION

High performance liquid chromatographic technique greatly enhanced the separation and quantification abilities in the bioactive peptides studies area [1 - 3]. Therefore, the measure of retention or mobility derived from this technique typically yield insufficient information in order to verify or quantify the presence of expected peptides

Three dimensional ancillary spectroscopic technique have made great contribution to the effectiveness of chromatographic system in a variety of application areas [4]. In recent years, one of them, HPLC combined with photodiode array detector technology, extended many of advances of ancillary spectroscopy in liquid chromatography [5-10]. The parallel detection nature of the

reverse optics diode array detection system provides the potential for absolute wavelength accuracy. This means that the UV spectra of even compounds with no prominent peaks in the UV spectra could be compared, and true differences resulting from the sample under study have been obtained [11 -12]. This technique has been successfully applied in peak purity verification and determination of aromatic amino acid content of protein and peptides [4].

We have previously reported isolation of two opioid peptides, LVV-hemorphin-7 and VV-hemorphin-7 from a very complex bovine hemoglobin peptic hydrolysate [13]. These isolated peptides or those obtained either from the bovine or human beta- chain of hemoglobin [14] contained important amounts of aromatic amino acids. This conferred peculiar spectra differing from that of other peptides. These properties allowed us to set up, using UV spectra comparison, a chromatographic method for identification of hemorphins from this peptic mixture. The aim of our study was to simplify the purification procedure of such expected bioactive peptides.

EXPERIMENTAL

MATERIALS AND CHEMICALS

All common chemicals and solvents were of analytical grade from commercial sources. Rabbit lung angiotensin converting enzyme (ACE) was purchased from Sigma Chemicals. Hemorphin-7 was synthesized by C.Guillon, Laboratoire de Technologie Enzymatique, University of Compiègne Compiègne, France

Hydrolysate, fraction F VII, LVV-hemorphin-7 and VV-hemorphin-7 preparation

Bovine hemoglobin hydrolysate was obtained at pilot- plant scale by peptic proteolysis in an ultrafiltration reactor as previously described [15]. Active fraction FVII was prepared by gel permeation HPLC using TSK G2000 SWG column (19mm i.d. X 600 mm) and analyzed by reversed phase (RP) HPLC in order to obtain LVV-hemorphin-7 and VV-hemorphin-7 as described previously [13].

LVV-hemorphin-5 and VV-hemorphin-5 preparation

1 mg of either VV-hemorphin-7 or LVV-hemorphin-7 were dissolved in 1 ml 0.05 M Tris - HCl buffer pH 7.4 and incubated at 37 °C with angiotensin converting enzyme (ACE) (7.5 mU) during 7 h. according to LANTZ [16]. The reaction mixture was resolved on a Nova-Pak C-18 column (3.9 mm i. d. X 150 mm). LVV-hemorphin-5 and VV-hemorphin-5 were identified by mass spectrometry.

HPLC system

The liquid chromatographic system consisted of Waters 600 automated gradient controller-pump module, Waters Wisp 717 automatic sampling device and Waters 996 photodiode array detector. Spectral and chromatographic data were

stored on NEC image 466 hard disc using NEC image 466 computer. Millennium software was used to plot, acquire and treat chromatographic data.

METHODS

Mobile phase for Delta Pak C-18 column (19 mm i.d.X 300 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 12 ml/min. Samples were dissolved in buffer A, filtered through 0.20 µm filters and injected. The gradient applied was 0-40% B in 80 min.

Mobile phase for Nova-Pak C-18 column (3.9 mm i. d. X 150 mm)

The mobile phase comprised: 10 mM ammonium acetate buffer pH 6.0 as eluent A and acetonitrile as eluent B. A linear gradient (15-30% B in 15 minute) was applied.

Procedure

Hemorphin-7, VV-hemorphin-7 and LVV-hemorphin-7 were chromatographed at room temperature on the Delta Pak C-18 and Nova Pak C-18 respectively with conditions described above. Total hydrolysate and fraction FVII were also injected on Delta Pak C-18 column under the same conditions. On-line instantaneous UV absorbance spectral scan were performed between 190 nm and 350 nm with a rate of one spectrum/second. Then the results of chromatographic analyses were completed by using Millennium software. Spectrum matching results (comparison spectra of the peaks in the chromatographic profile with library spectra of the hemorphins.) were reported by Waters Millenium system as:

Match angle - A measure of the difference in spectral shapes between an unknown spectrum and a library spectrum. Match angle can range from 0 to 90 degrees. Lower values indicate that spectra are similar. Larger values indicate greater degrees of spectral difference.

Match threshold - Sensibility of the measurement. It can range from 0 to 180 degrees. Larger values indicates a lower sensibility of the measurement. In general, if the match angle is greater than the match threshold, it indicates that two spectra are different. If the match angle is less than the match threshold, it does not indicate that the two spectra are different.

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. 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. [17]. The detection wavelength was 254 nm and the flow rate 1 ml/min.

Mass spectrometry analysis

Mass spectra, generated from Fast Atom Bombardment (FAB) mass spectrometry of the peptides, 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 8 KeV. The peptide was dissolved in water (1 µg/µl) and 1µl 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.

RESULTS AND DISCUSSION

COMPARISON OF HEMORPHINS SPECTRA

Hemorphin-7 (50 µg), VV-hemorphin-7 (50 µg), LVV-hemorphin-7 (15 µg), or a mixture of VV-hemorphin-7 (50 µg) or LVV-hemorphin-7 (50 µg) incubated with ACE (15) were successively loaded on the Nova Pak C18 column.

The UV spectra of protein and peptides are greatly dependent on their amino acids content. Unlike aromatic amino acids (phenylalanine, tryrosine and tryptophan) the others have very similar spectra. Only the aromatic amino acids exhibit maximal absorbances between 250 and 300 nm, nevertheless, their spectra are broad and they overlap.

Absorbance UV spectra of hemorphins are shown in figure 1. They were very similar especially between 250 and 300 nm. This was due to the same amount of Try and Trp in these molecules. Only the mathematical analysis and comparison of spectra allowed to differentiate them. The differences between spectra are displayed in table 1. When one spectrum was matched with itself, match angle was zero. The smaller match angle indicated that two molecules were almost similar. For example, as far as LVV-hemorphin-7 was concerned, it matched more or less with the other hemorphins. Thus, match angle values could be classified following an increasing order: LVV-hemorphin-7 < VV-hemorphin-7 < hemorphin-7 < LVV-hemorphin-5 < VV-hemorphin-5. It confirmed that, regarding hemorphins primary structure, the degree of similarity were inversely related with match angle values.

It could be noticed that hemorphin-7 and VV-hemorphin-5, in spite of the same chain length, showed different match angles when compared with LVV-hemorphin-7. According to the match angle values, hemorphin-7 was much similar to LVV-hemorphin-7 than VV-hemorphin-5. So, when peptides similarity is required, if aromatic amino acids seems to prevail, the chain length should also be considered (Table 2)

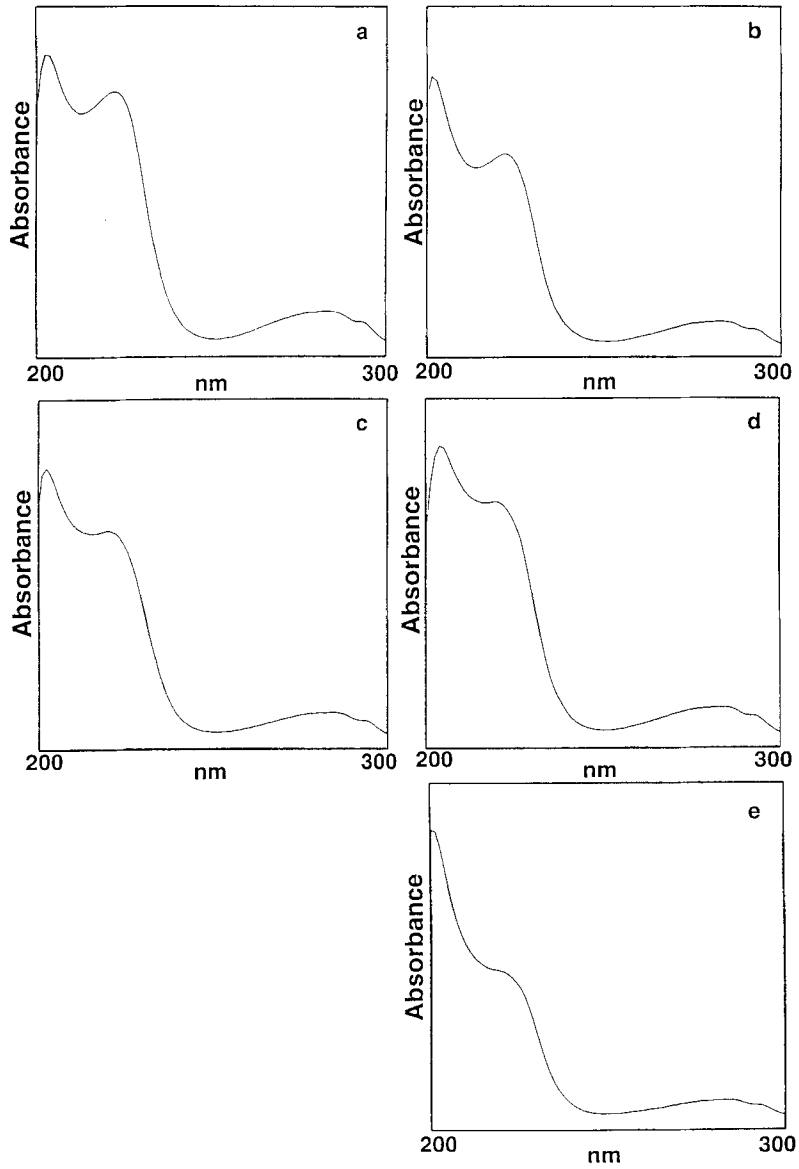

Figure 1: UV-spectra of the hemorphins obtained by photodiode array detector during their chromatographic analyses on a Nova Pak C18 column with the conditions described in experimental section. a) VV-hemorphin-5; b) LVV-hemorphin-5; c) Hemorphin-7; d) VV-hemorphin-7; e) LVV-hemorphin-7.

TABLE 1
Match Angle (MA) and Match Threshold (MT) Calculated by Millenium System from the Comparison of UV Spectra of Hemorphins during Chromatographic Analyses on Nova Pak Column.

Library spectra	VV-hemorphin-5		LVV-hemorphin-5		Hemorphin-7		VV-hemorphin-7		LVV-hemorphin-7	
Sample spectra	MA	MT	MA	MT	MA	MT	MA	MT	MA	MT
VV-hemorphin-5	0.00	0.051	0.784	0.073	2.695	0.045	3.366	0.212	3.175	0.181
LVV-hemorphin-5	0.784	0.073	0.00	0.084	1.583	0.066	2.748	0.219	2.694	0.187
Hemorphin-7	2.693	0.045	1.581	0.065	0.00	0.034	1.466	0.021	1.717	0.179
VV-hemorphin-7	3.366	0.212	2.748	0.219	1.466	0.211	0.00	0.288	0.559	0.274
LVV-hemorphin-7	3.175	0.182	2.693	0.188	1.717	0.179	0.559	0.274	0,00	0,244

TABLE 2
Primary Structure of Hemorphins

Hemorphins	Structure									
VV-hemorphin-5		Val	Val	Tyr	Pro	Trp	Thr	Gln		
LVV-hemorphin-5	Leu	Val	Val	Tyr	Pro	Trp	Thr	Gln		
Hemorphin-7				Tyr	Pro	Trp	Thr	Gln	Arg	Phe
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

VERIFICATION OF THE METHOD RELIABILITY

The method was tested by searching VV-hemorphin-7 and LVV-hemorphin-7 in fraction FVII of the hemoglobine hydrolysate by use of UV spectral comparison

Hemorphin-7 (50 µg), VV-hemorphin-7 (50 µg) and LVV-hemorphin-7 (15µg) were injected on a Delta Pak column under the conditions described in experimental section. Chromatographic profiles and UV spectra of hemorphins were recorded (Fig. 2). With photodiode array detector, two chromatographic parameter were obtained in the same time: retention time and real time spectra. Then, fraction FVII was injected on the same column under the same conditions. Figure 3 exhibits its chromatographic profile. The hemorphins UV spectra were matched with every peak in this fraction. Table 3 exhibited the results. When we matched VV-hemorphin-7 UV-spectrum as library spectrum towards all the peaks of the profile, peak 3 was identified as VV-hemorphin-7. In the same manner, peak 4 was identified as LVV-hemorphin-7. This corroborated the results obtained with the same column from previous studies involving time consuming purification steps [14].

Verification was also carried out by use of hemorphins UV spectra obtained from Nova Pak column. Under these conditions it was impossible to detect VV-hemorphin-7 and LVV-hemorphin-7 only by use of their retention time. Therefore, matching of their spectra could bring more informations. The results are presented

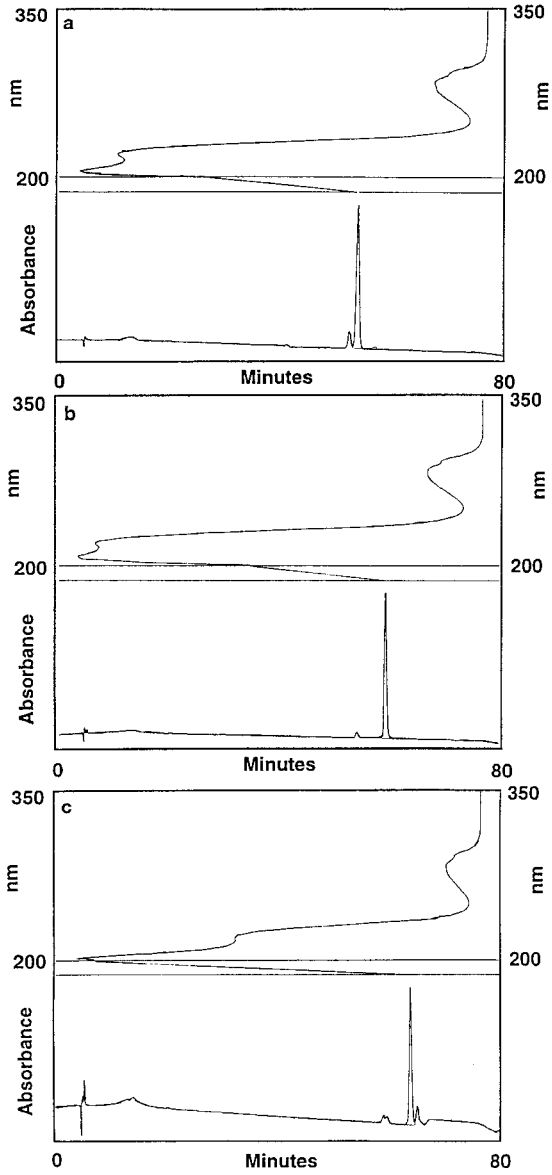

Figure 2: UV-spectra and correspondent chromatographic profiles of the hemorphins obtained by a photodiode array detector and a HPLC Delta Pak C18 column with the conditions described in experimental section. a) Hemorphin-7; b) VV-hemorphin-7; c) LVV-hemorphin-7.

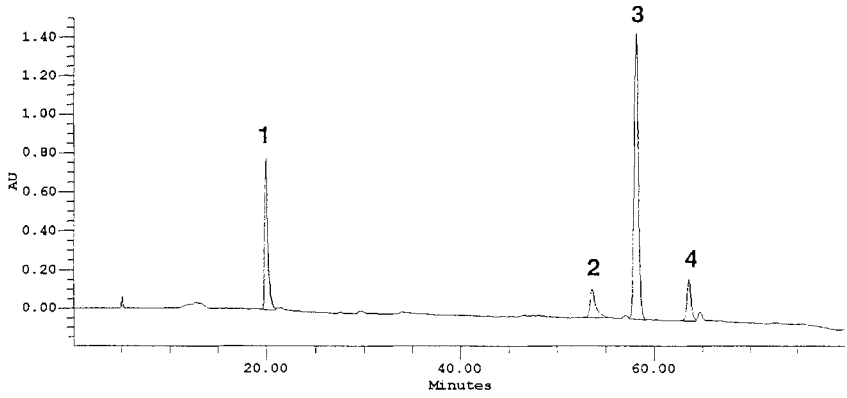

Figure 3: Chromatographic profile of fraction FVII, issued from the TSK G2000 SWG column, on a Delta Pak C18 column with the conditions described in experimental section. Peak 3 and 4 were identified as VV-hemorphin-7 and LVV-hemorphin by UV-spectra comparison.

TABLE 3
Spectral Matching Results of the Hemorphins with the Peaks of Fraction VII (fig.3) Separated by Delta Pak C18 Column. Peak 1 and 2 Were Rejected. Peak 3 and 4 Were Identified as VV-Hemorphin 7 and LVV-Hemorphin 7 Respectively Match Angle (MA), Match Threshold (MT)

Library spectra	Hemorphin-7		VV-hemorphin-7		LVV-hemorphin-7	
Sample spectra	MA	MT	MA	MT	MA	MT
Peak 3	1.754	1.003	**0.551**	**1,004**	1.330	1.006
Peak 4	2.829	1.013	1.950	1.014	**0.148**	**1.103**

in Table 4. Peak 3 and peak 4 were identified as VV-hemorphin-7 and LVV-hemorphin-7 by their match angles. So, it became an evidence that matching spectra were not limited to strict conditions: whatever the column, the method was still efficient.

IDENTIFICATION OF VV-HEMORPHIN-7 AND LVV-HEMORPHIN-7 FROM A COMPLEX PEPTIC BOVINE HEMOGLOBIN HYDROLYSATE

The previous experiments represented analyses performed on a simple fraction. Our purpose was to know whether it was possible to extract similar information from a more complex hydrolysate. So, peptic bovine hemoglobin hydrolysate (10 mg) was loaded on a Delta Pak C18 column. The profile is presented in figure 4. About 60 peaks could be resolved. Each peak was matched with UV spectra of hemorphins obtained from Delta Pak column. The results are shown in Table 5. Among 60 peaks, only 4 were selected by this calculation (peaks 27, 41, 51, 52), the other peaks exhibited match angles greater than than 90

TABLE 4
Spectral Matching Results of the Hemorphins with the Peaks of Fraction VII (fig.3) Separated by Nova Pak column. Peak 1 and 2 Were Rejected. Peak 3 and 4 Were Identified as VV-Hemorphin 7 and LVV-Hemorphin 7 Respectively. Match Angle (MA), Match Threshold (MT)

Library spectra	VV-hemorphin-5	LVV-hemorphin-5	Hemorphin-7	VV-hemorphin-7	LVV-hemorphin-7
Sample spectra	MA	MA	MA	MA	MA
Peak 3	2.643	2.051	1.063	0.51	1.677
Peak 4	4.394	3.332	2.794	3.332	0.562

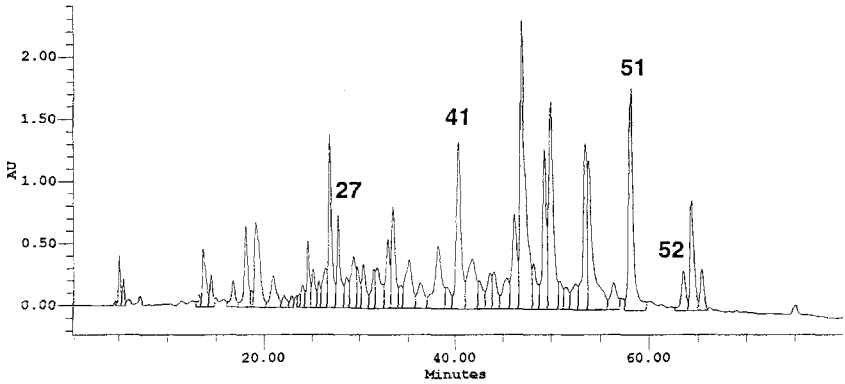

Figure 4: Chromatographic profile of on a Delta Pak C18 column with the conditions described in experimental section. Peaks 27, 41, 51 and 52 were selected by matching spectra with hemorphins. Peak 51 and 52 were identified as VV-hemorphin-7 and LVV-hemorphin-7.

degrees. It signified that their UV spectra were incomparable with those of hemorphins, probably because their primary structure were quite different from the hemorphins. When LVV-hemorphin-7 was used as library spectrum, the peak 52 was identified as LVV-hemorphin-7. In the same way, peak 51 was identified as VV-hemorphin-7. When hemorphin-7 was used as library spectrum, the minimum match angle was peak 51, identified as VV-hemorphin-7. It indicated that, as already described, VV-hemorphin-7 was found the most similar molecule to hemorphin-7 (Table 1). Consequently, hemorphin-7 was not present in total hydrolysate.

We used also the UV-spectra of the hemorphins obtained from Nova Pak column as library spectra. Similar results were obtained. So, the procedure of purification of LVV-hemorphin-7 and VV-hemorphin-7 was greatly simplified by only

TABLE 5
Spectral Matching Results of the Hemorphins with All the Peaks of the Bovine Hemoglobin Hydrolysate (fig.4).Separated by Delta Pak C18 Column. Peak 27, 41, 51, 52 Were Selected. Peak 51 and 52 Were Identified as VV-Hemorphin 7 and LVV-Hemorphin 7 Respectively. Match Angle (MA), Match Threshold (MT)

Library spectra	Hemorphin-7		VV-hemorphin-7		LVV-hemorphin-7	
Sample spectra	MA	MT	MA	MT	MA	MT
Peak 27	9.196	0.005	9.811	1,006	9.612	1.006
Peak 41	4.956	0.003	6.093	1.003	5.236	1.103
Peak 51	0.824	0.003	0.398	1.004	1.707	1.008
Peak 52	2.856	0.012	2.999	1.012	0.332	1.024

IDENTIFICATION OF VV-HEMORPHIN-7 AND LVV-HEMORPHIN-7 BY BIOCHEMICAL ANALYSIS

By analysis of amino acid composition, FAB mass spectrometry and in regard of alpha and beta chains of bovine hemoglobin, the sequences of the four peaks selected among 60, were found out as following:

P27: Asn - Val - Lys - Ala - Ala - Trp - Gly - Lys - Val
(9 -17 of alpha chain of bovine hemoglobin)
P41: Ser - Ala - Ala - Asp - Lys - Gly - Asn - Val - Lys - Ala - Ala - Trp
(3 - 14 of alpha chain of bovine hemoglobin)
P51: Val - Val - Tyr - Pro - Trp - Thr - Gln - Arg - Phe
(32 - 40 of beta chain of bovine hemoglobin, **VV-Hemorphin 7**)
P52: Leu - Val - Val - Tyr - Pro - Trp - Thr - Gln - Arg - Phe
(31 - 40 of beta chain of bovine hemoglobin, **LVV-Hemorphin 7**)

As a result, peaks 51 and 52 were identified as VV-hemorphin-7 and LVV-hemorphin-7. These results validated the results obtained by matching spectra. We may suppose that Peaks 27 and 47 were retained by the spectral detection owing to their tryptophan content. Meanwhile, their match angle values were too much high to put them in the same category as hemorphins. Trp seemed to play a great role in spectrum matching because other peptides containing other aromatic amino acids, which are numerous in hemoglobin hydrolysate [18-19], were not assimilated to hemorphins by match spectra.

In this work, a rapid method for identification of hemorphins issued from a complex bovine hemoglobin hydrolysate was established. Its accuracy was verified by biochemical analysis. As mentioned above, aromatic amino acids played a very important role in matching spectra. This property could also be applied to the

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