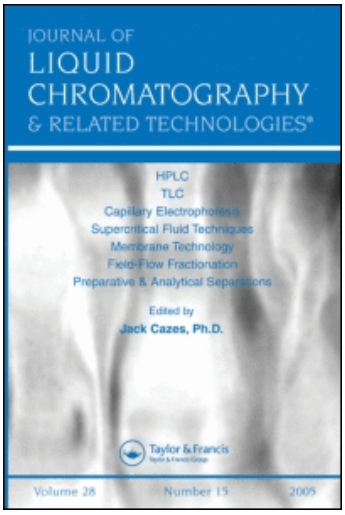

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Peptic Peptide Mapping by HPLC, on Line With Photodiode Array Detection, of a Hemoglobin Hydrolysate Produced at Pilot-Plant Scale from an Ultrafiltration Process

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PEPTIC PEPTIDE MAPPING BY HPLC, ON LINE WITH PHOTODIODE ARRAY DETECTION, OF A HEMOGLOBIN HYDROLYSATE PRODUCED AT PILOT-PLANT SCALE FROM AN ULTRAFILTRATION PROCESS

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ABSTRACT

The analysis of a peptic bovine hemoglobin hydrolysate, produced at pilot plant scale, was carried out using two techniques: SE-HPLC and RP-HPLC. Analysis of amino acid composition, second-order derivative spectrometry and FAB mass spectrometry of isolated peptides allowed us to determine the exact positions of these peptides in the sequence of bovine hemoglobin. This, consequently, gave rise to a peptidic map of the hydrolysate. It also revealed, at the same time, some biologically active peptides in the hydrolysate. This information should find use in the potential future application of enzymatic bovine hemoglobin hydrolysate.

INTRODUCTION

Enzymatic hydrolysis of proteins frequently destroys their functionality. In some cases, however, proteolysis is useful for improving their functional properties, since it gives rise to peptide mixtures which generate many potential applications such as culture media,[1] in stimulating fermentation,[2] nutritional therapy,[3] and research focused on finding peptides possessing biological activity.[4] In this area enzymatic hydrolyses of casein,[5] soya proteins,[6] gluten,[7] tuna myoglobin,[8] and bovine hemoglobin[9] were investigated.

Concerning hemoglobin, extensive research has been carried out investigating some endogenous substances to their opioid activity or affinity for opioid receptors.[10-13] Indeed, many fragments of hemoglobin were isolated from various organs exihibiting potential biological activity.[14,15] These results indicate that hemoglobin could be a precursor of many biologically active peptides.

The peptic hydrolysis of bovine hemoglobin, which is a source of high annual industrial waste, was carried out in an ultrafiltration reactor at pilot plant scale.[9] This experimental set up allowed us to obtain a well-defined and very reproducible peptic hydrolysate from bovine blood hemoglobin.[16]

Some biologically active peptides such as opioid peptides,[17] bradykinin potentialisating peptide,[18] and bacteria growth stimulating peptide[19] were also isolated. This hydrolysate has also been shown to have growth stimulating properties in fermentation.[20]

In this study, we isolated and identified all of the peptides present in the hydrolysate. Peptide fractionation and purification were performed by using reverse-phase (RP) HPLC to complement the size exclusion (SE) HPLC separation and the amino acid composition of each peptide analysed. The masses of some of the peptides were confirmed by fast atom bombardment mass spectrometry (FABMS). At the same time, a second-order derivative spectrometry technique[21] was used to determine the presence of aromatic amino acids in the peptide. This was particularly useful for peptides containing tryptophan, since these are destroyed during amino acid analysis.

The identification and location of the different peptides in the known bovine hermoglobin structure were carried out; this resulted in a peptidic map of this hydrolysate. Such a peptide map provides important information for potential future applications.

MATERIALS AND METHODS

All common chemicals and solvents were of analytical grade from commercial sources. Acetonitrile was of HPLC grade. Water was obtained from a Waters (Saint-Quentin, France) Milli-Q system. The amino acids standard kit H was from Pierce Chemical Co. Porcine pepsin was purchased from Sigma Chemicals. All aqueous HPLC eluents were degassed with helium (Air Liquide, La Rochelle, France) during analysis.

Peptic Hemoglobin Hydrolysis

Decolorized bovine hemoglobin hydrolysate was obtained at pilot-plant scale by peptic proteolysis in an ultrafiltration reactor, followed by decolorization with magnesia, desalting and atomization as previously described.[9] The nitrogen content, determined by the Kjeldahl method, allowed evalution of the quantity of peptide in the hydrolysate greater than 90% (N X 6.25).

HPLC System

The liquid chromatographic system consisted of a Waters 600E automated gradient controller-pump module, a Waters WISP 717 automatic sampling device and a Waters 996 photodiode array detector. Spectral and chromatographic data were stored on a NEC image 466 computer. Millennium software was used to plot, acquire and analyze chromatographic data.

SE-HPLC

The elutions were performed with a TSK G2000SWG column (300 mm x 21.9 mm i.d.) with 7 mM ammonium acetate. Hydrolysate powder samples (400 mg) were dissolved in 2 mL of the same solution and filtered through 0.22 μm filters before being applied to the column. The flow rate was 5 mL/min. Fractions were collected and freeze dried immediately.

RP-HPLC

Analysis of the peptide fractions eluted from the TSK G2000SWG were carried out using a Delta Pak C-18 column (300 mm X 3.9 mm i.d.). 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. Samples were dissolved in buffer A, filtered through 0.22 μm filters and then injected. The gradient applied was 0-40% B over an 80 min period for fractions F I, F II and F III, 0-20% B over 40 min then 20-40% B over 20 min for fractions F IV, F V, F VI and F VII. On-line, instantaneous UV absorbance spectral scans were performed between 200 nm and 300 nm at a rate of one spectrum/second. The resolution was 1.2 nm. Chromatographic analysis was completed using Millennium software.

Amino Acid Analysis

Amino acids were analyzed using a Waters Picotag Work Station. Peptide hydrolysis was achieved by constant-boiling of the peptides with HCl (6N) which contained 1% phenol, for 24 h. at 110 °C. Precolumn derivatization of amino acids with phenyl isothiocyanate and HPLC separation of derivatized amino acids were performed on a Waters RP-Picotag column (150 mm x 3.9 mm i.d.) according to Bidlingmeyer et al.[22] The detection wavelength was 254 nm and the flow rate 1 mL/min.

Fast Atom Bombardment Mass Spectrometry

A Kratos MS 50 RF high resolution mass spectrometer, equipped with a DS 90 (DGDG/30) data system, was used. The mass spectrometer was operated at an 8-keV accelerating potential. An Ion Tech Model B 11 NF saddle field fast atom source energized with the B 50 current-regulated power supply was used with xenon as the bombarding atom (operating condition: 7.3 kV, 1.2 mA). Peptides were dissolved in water (250 mg in 50 mL) and 1 mL of the solution was loaded onto the copper tip with thioglycerol as a matrix. In this case, the source housing was not heated. The mass range was scanned at 10 s/decade with a mass resolution of 3000. Caesium iodide was the calibration standard.

RESULTS AND DISCUSSION

SE-HPLC

According to the HPLC gel filtration conditions described above, 30 mg/150μL of bovine hemoglobin hydrolysate could be loaded per run. The elution profile is shown in Figure 1. In this figure, seven fractions (F I to F

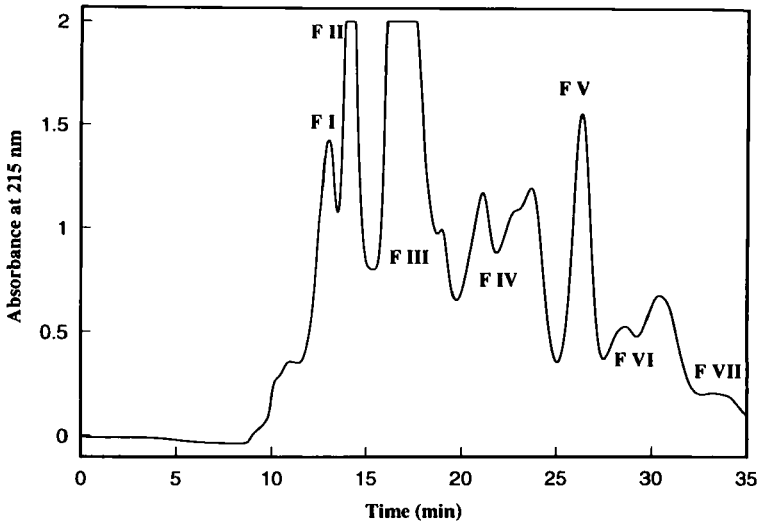

Figure 1. Elution profile of total peptic hydrolysate on TSK G2000 SWG column (300 mm x 21.5 mm i.d.) with 7 mM ammonium acetate . Flow rate is 6mL/min. Quantity injected for each run was 30 mg/150 µL.

VII) can be seen. It appears, from this profile, that the resolution of the sample components could be achieved in less than 35 min. with a similar efficiency as observed in the analytical separation.[23] Such separations result in easy collection of any fraction. More than seventy elutions were performed and, in each case, the same elution pattern was found. This led us to purify the largest quantity of material available for further RP-HPLC analysis or experimental application.

Reversed-Phase HPLC

Each fraction eluted from TSK G2000 SWG column was analysed by RP-HPLC with a Delta Pak C18 column. In order to improve optimal separation conditions of peptides having very different hydrophobicities, the gradients (acetonitrile / ammonium acetate) applied were altered for each fraction. This indicated that FI to FIV were relatively hydrophilic in comparison to FV to FVII. This may also be an important factor in the separation of hydrolysates by SE-HPLC. Indeed, many factors, such as hydrophobicity and electric charge, can affect the behavior of the peptides during size exclusion chromatography.[24] The chromatograms issued from the RP-HPLC separation are presented in

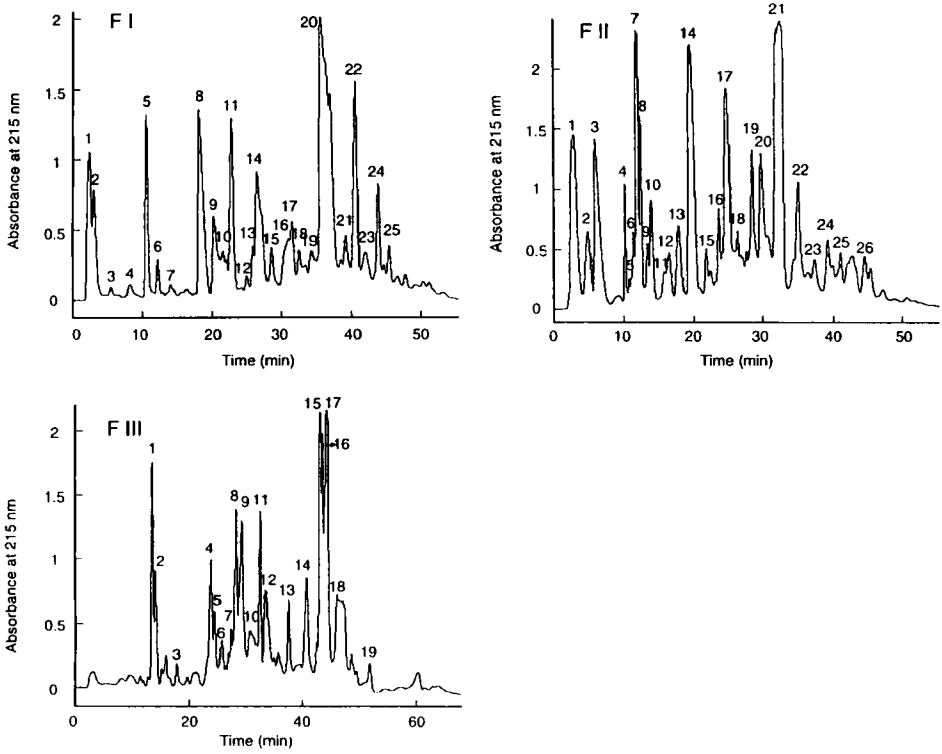

Figure 2. RP-HPLC on Delta Pak -C18 column (300 mm X 3.9 mm i. d.) of peptide fractions F I to III eluted from TSK G2000 SWG column. The mobile phase comprised 10 mM amonium acetate buffer pH 6.0 as eluent A and acetonitrile as eluent B. The flow rate was 1.5 mL/min. Injected quantity was 1.5 mg / 100 μL. The gradient applied was 0-40% B in 80 min .

Figure 2 and Figure 3. From these profiles, an excellent resolution of the peptide mixtures for each fraction was obtained. Therefore, the individual peaks were collected separately and they were labelled according to the process of isolation. Each peak was checked for its purity by RP-HPLC. In total, 131 peptides were collected and freezed-dried before amino acid analysis.

Second-Order Derivative Spectrometry

Before amino acid analysis, the secondary derivative spectrum of each peptide was obtained. As previously reported,[21] HPLC, coupled with a

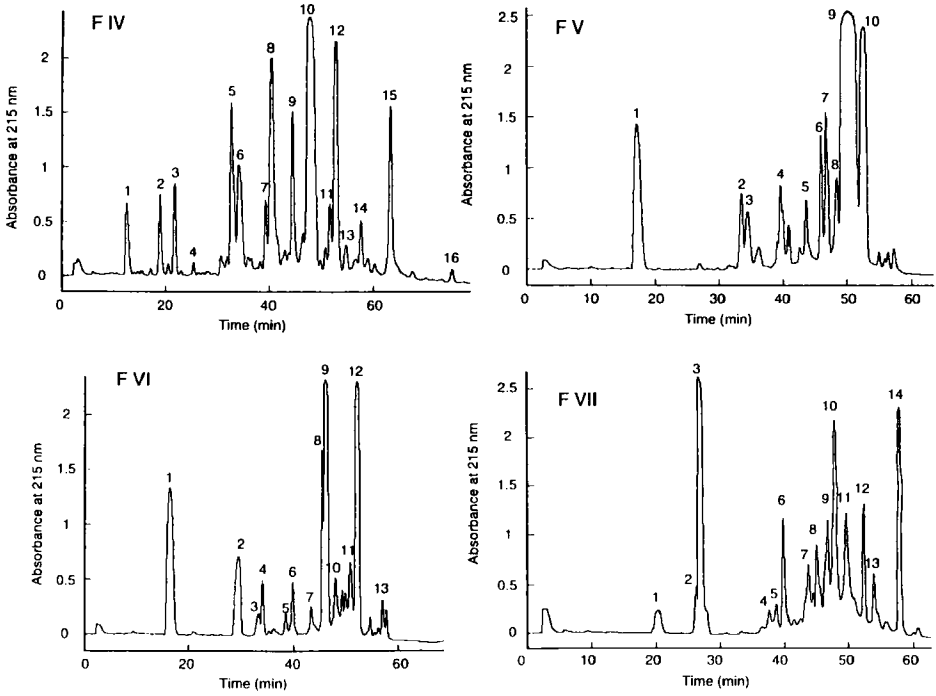

Figure 3. RP-HPLC on Delta Pak -C18 column (300 mm X 3.9 mm i. d.) of peptidic fractions (F IV to VII) eluted from TSK G2000 SWG column. The mobile phase comprised 10 mM amonium acetate buffer pH 6.0 as eluent A and acetonitrile as eluent B. The flow rate was 1.5 mL/min. Injected quantity was 1.5 mg / 100 µL. The gradient applied was 0-20% B in 40 min then 20 to 40% in 20 min for IV to VII.

photodiode array detector, allowed us to obtain the UV spectrum of each peak, instantaneously, during the chromatographic separation. The second-order derivative spectrum obtained from the UV spectrum of each peak was used for the detection of aromatic amino acids in the peptide. Figure 4 presents the second-order derivative spectra of peptides F I-8, F V-1, F II-19 and F V-10. The presence of Phe, Tyr, Trp in F I-8, F V-1, F II-19, respectively, were indicated by minima at 258.5 nm, 283.5 nm, and 289.5 nm. F V-10 contained three aromatic amino acids as was previously found.[21] Therefore, the second-order derivative spectrum of each peptide was studied and the presence/absence of aromatic amino acids in each peptide determined; these results are shown in Table 1. This was very useful for the peptides containing tryptophan, since it is destroyed during amino acid analysis and was also an important marker in the subsequent sequence analysis of each peptide.

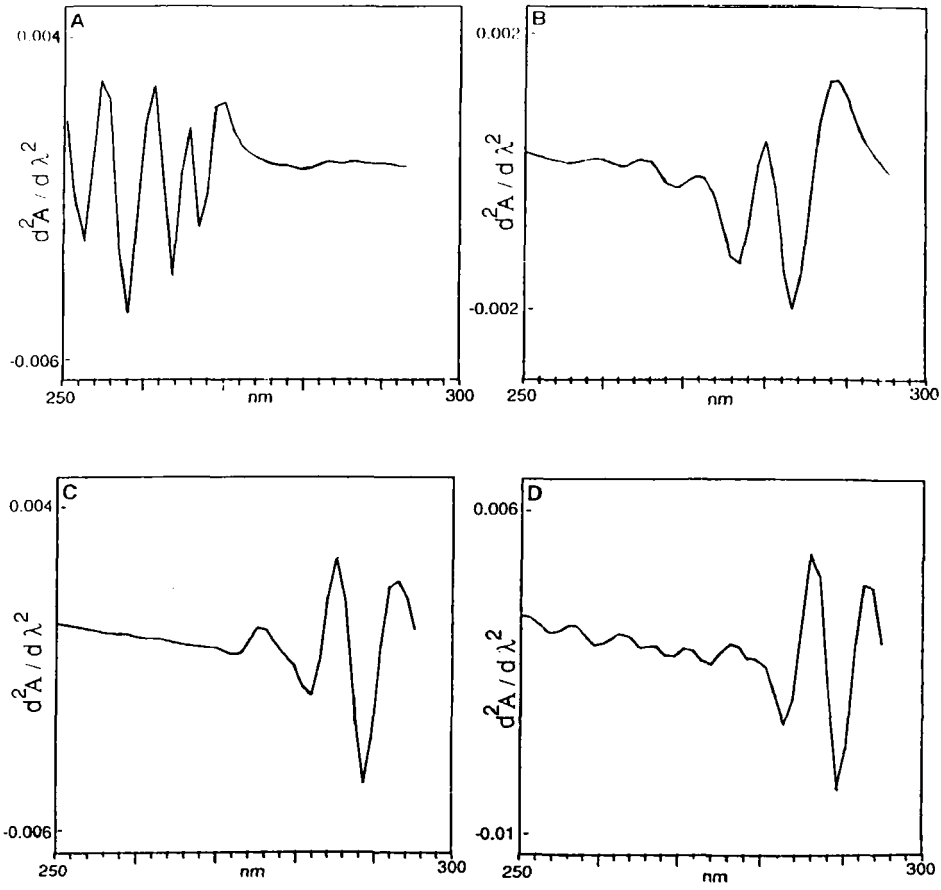

Figure 4. Second-order derivative spectra of peptides F I-8 (a), F V-1 (b), F II-19 (c) and F V-10 (d). The presence of Phe, Tyr, Trp in F I-8, F V-1, F II-19, respectively, was indicated by the absorbance minima at 258.5, 283.5, and 289.5 nm. F V-10 contained three aromatic amino acids.

Amino Acid Analysis

All isolated peptides were analyzed on the Waters Pico-Tag column after converting amino acids into their PTC (phenylthyocarbamyl) derivatives. For example, Figure 5 shows the chromatographic profile of peptide F V-1. In comparison with that of standards, this led us to determine the composition of this peptide as: Ser (1), Arg (1), Thr (1), Tyr (1) and Lys (1). With regard to

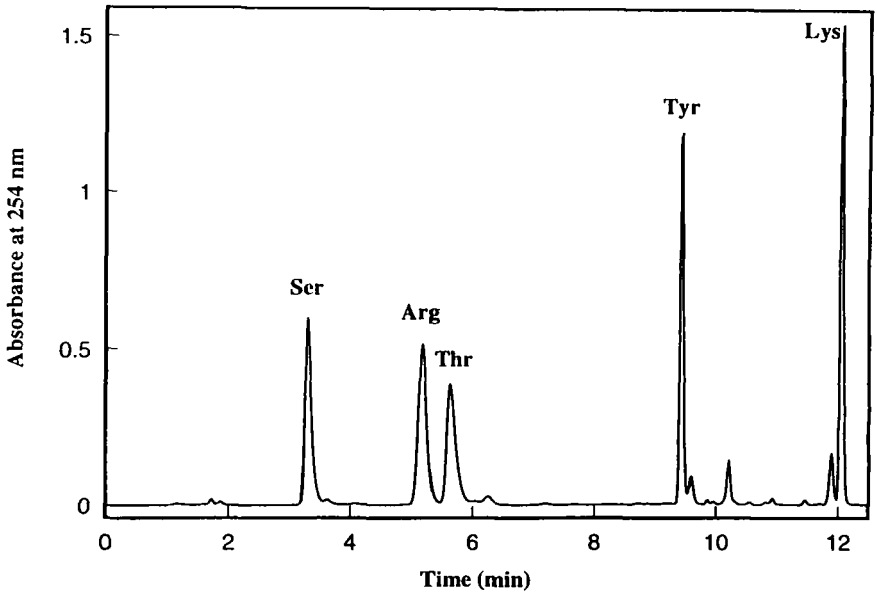

Figure 5. Elution profile of PTC amino acid of peptide F V-5 obtained by HPLC with Waters picotag column. The amino acid composition of this peptide was deduced as: Ser (1), Arg (1) Thr (1), Tyr (1) Lys (1).

the known sequence of bovine hemoglobin, this peptide was determined as fragment 137-141 of the α chain of bovine hemoglobin. In the same way, all the peptides were localized and represented in Table 1. A peptidic map of this hydrolysate could then be constructed as shown in Figure 6 and Figure 7. It was observed that some peptides, issued from different fractions of the SE-HPLC separation, had an identical sequence (F I-9 = F II-11; F V-1 = F VI-1; FVI-3 = F VII-1, etc). These results were also verified by a UV-spectral comparison technique.[25] From this, we observed that the SE-HPLC was only a preliminary separation, and it was possible that the same peptide was present in different fractions. It was also noticed that some peptides issued from the same zone of α or β chains were eluted in the same fraction from the SE-HPLC separation by a TSK G2000 SWG column. For example: many peptides from fraction I or Fraction II were localised in the zone 65-85 of α chain of hemoglobin. The two major peaks from fraction V-9 and V-10 were issued from the region 31-40 of β chain of hemoglobin. It was also observed that almost all of the peptides from fraction V, fraction VI and fraction VII contained aromatic amino acids. These phenomena were also found in the

Table 1

Identification of Peptides from a Bovine Hemoglobin Peptic Hydrolysate Isolated by SE-HPLC Followed by RP-HPLC

Fraction	Peptide No.	Corresp. Fragment	Sequence	Aromatic Amino Acid
I	I-1	3-6 β	Thr-Ala-Glu-Glu	
	I-2	126-128 β	Leu-Gln-Ala-Asp	
	I-3	20-21 β	Asp-Glu; Glu-Asn (100-101 β); Asn-Glu (119-120 β)	
	I-4	2-5 β	Leu-Thr-Ala-Glu	
	I-5	96-101 β	His-Val-Asp-Pro-Glu-Asn	
	I-6	45-51 β	Gly-Asp-Leu-Ser-Thr-Ala-Asp	
	I-7	2-6 β	Leu-Thr-Ala-Glu-Glu	
	I-8	127-130 β	Ala-Asp-Phe-Gln	Phe
	I-9	1-6 β	Met-Leu-Thr-Ala-Glu-Glu	
	I-10	82-85 α	Glu-Leu-Ser-Asp	
	I-11	93-102 β	Asp-Lys-Leu-His-Val-Asp-Pro-Glu-Asn-Phe	Phe
	I-12	125-128 β	Leu-Gln-Ala-Asp	
	I-13	42-47 β	Glu-Ser-Phe-Gly-Asp-Leu	Phe
	I-14	74-80 α	Asp-Asp-Leu-Pro-Gly-Ala-Leu,	
		73-79 α	Leu-Asp-Asp-Leu-Pro-Gly-Ala	
	I-15	71-75 α	Glu-His-Leu-Asp-Asp	
	I-16	68-78 α	Lys-Ala-Val-Glu-His-Leu- Asp-Asp-Leu-Pro-Gly	
	I-17	93-100 β	Asp-Lys-Leu- His-Val-Asp-Pro-Glu	
	I-18	42-48 β	Glu-Ser-Phe-Gly-Asp-Leu-Ser	Phe
	I-19	72-80 α	His-Leu-Asp-Asp-Leu-Pro-Gly-Ala-Leu	
	I-20	41-47 β	Phe-Glu-Ser-Phe-Gly-Asp-Leu	Phe
	I-21	100-105 β	Glu-Asn-Phe-Lys-Leu-Leu	Phe
	I-22	65-78 α	Ala-Leu-Thr-Lys-Ala-Val-Glu-His-Leu-Asp-Asp-Leu-Pro-Gly	
		67-80 α	Thr-Lys-Ala-Val-Glu-His-Leu-Asp-Asp-Leu-Pro-Gly-Ala-Leu	
	I-23	67-82 α	Thr-Lys-Ala-Val-Glu-His-Leu-Asp-Asp-Leu-Pro-Gly-Ala-Leu-Ser-Glu	
	I-24	66-80 α	Leu-Thr-Lys-Ala-Val-Glu-His-Leu-Asp-Asp-Leu-Pro-Gly-Ala-Leu	
	I-25	68-80 α	Lys-Ala-Val-Glu-His-Leu-Asp-Asp-Leu-Pro-Gly-Ala-Leu	

Table 1 (continued)

Identification of Peptides from a Bovine Hemoglobin Peptic Hydrolysate Isolated by SE-HPLC Followed by RP-HPLC

Fraction	Peptide No.	Corresp. Fragment	Sequence	Aromatic Amino Acid
II	II-1	48-52 β	Ser-Thr-Ala-Asp-Ala	
	II-2	45-49 β	Gly-Asp-Leu-Ser-Thr	
	II-3	80-82 α	Leu-Ser-Glu, Ser-Glu-Leu (81-83 α), Glu-Leu-Ser (82-84 α)	
	II-4	95-101 β	Leu-His-Val-Asp-Pro-Glu-Asn	
	II-5	27-29 α	Glu-Ala-Leu, Ala-Leu-Glu (28-30 α)	
	II-6	125-131 β	Leu-Gln-Ala-Asp-Phe-Gln-Lys	Phe
	II-7	22-27 α	Ala-Glu-Tyr-Gly-Ala-Glu, Glu-Tyr-Gly-Ala-Glu-Ala (23-28 α)	Tyr
	II-8	22-28 α	Ala-Glu-Tyr-Gly-Ala-Glu-Ala	Tyr
	II-9	22-27 β	Val-Gly-Gly-Glu-Ala-Leu	
	II-10	126-130 β	Gln-Ala-Asp-Phe-Gln	Phe
	II-11	1-6 β	Met-Leu-Thr-Ala-Glu-Glu	
	II-12	81-85 α	Ser-Glu-Leu-Ser-Asp	
	II-13	2-7 β	Leu-Thr-Ala-Glu-Glu-Lys	
	II-14	24-29 α	Tyr-Gly-Ala-Glu-Ala-Leu	Tyr
	II-15	73-79 α	Leu- Asp-Asp-Leu-Pro-Gly-Ala	
	II-16	89-94 β	Glu-Leu-His-Cys-Asp-Lys	
	II-17	41-44 β	Phe-Glu-Ser-Phe; Phe-Phe-Glu-Ser (40-43β)	Phe
	II-18	125-128 β	Leu-Gln-Ala-Asp	
	II-19	14-21 β	Trp-Ser-Lys-Val-His-Val-Asp-Glu	Trp
	II-20	42-47 β	Glu-Ser-Phe-Gly-Asp-Leu	Phe
	II-21	14-28 β	Trp-Ser-Lys-Val-His-Val-Asp-Glu-Val-Gly-Gly-Glu-Ala-Leu-Gly	Trp
	II-22	67-80 α	Thr-Lys-Ala-Val-Glu-His-Leu-Asp-Asp-Leu-Pro-Gly-Ala-Leu	
		66-79 α	Leu-Thr-Lys-Ala-Val-Glu-His-Leu-Asp-Asp-Leu-Pro-Gly-Ala	
		65-78 α	Ala-Leu-Thr-Lys-Ala-Val-Glu-His-Leu-Asp-Asp-Leu-Pro-Gly	
	II-23	110-120 α	Ala-Ser-His-Leu-Pro-Ser-Asp-Phe-Thr-Pro	
	II-24	109-120 α	Leu-Ala-Ser-His-Leu-Pro-Ser-Asp-Phe-Thr-Pro	
	II-25	124-128 β	Val-Leu-Gln-Ala-Asp	
	II-26	124-129 β	Val-Leu-Gln-Ala-Asp-Phe	

(continued)

Table 1 (continued)

Identification of Peptides from a Bovine Hemoglobin Peptic Hydrolysate Isolated by SE-HPLC Followed by RP-HPLC

Fraction	Peptide No.	Corresp. Fragment	Sequence	Aromatic Amino Acid
III	III-1	97-99 α	Asn-Phe-Lys; Asp-Lys-Phe (126-128 α)	Phe
	III-2	84-87 β	Phe-Ala-Ala-Leu	Phe
	III-3	30-32 α	Glu-Arg-Met	
	III-4	134-136 α	Thr-Val-Leu; Val-Leu-Thr (135-137 α); Val-Thr-Leu(107-109; α); Leu-Val-Thr (106-108, α); The-Leu-Val (105-107 α)	
	III-5	48-56 α	Leu-Ser-His-Gly-Ser-Ala-Gln-Val-Lys	
	III-6	24-34 α	Tyr-Gly-Ala-Glu-Ala-Leu-Glu-Arg-Met-Phe-Leu	Tyr, Phe
	III-7	124-128 β	Val-Leu-Gln-Ala-Asp	
	III-8	123-138 β	Pro-Val-Leu-Gln-Ala-Asp-Phe-Gln-Lys-Val-Val-Ala-Gly-Val-Ala-Asn	Phe
	III-9	127-134 α	Lys-Phe-Leu-Ala-Asn-Val-Ser-Thr	Phe
	III-10	105-107 α	Leu-Leu-Val	
	III-11	33-35 α	Phe-Leu-Ser; Leu-Ser-Phe (34-36 α)	Phe
	III-12	130-140 α	Ala-Asn-Val-Ser-Thr-Val-Leu-Thr-Ser-Lys-Tyr	Tyr
	III-13	129-134 α	Leu-Ala-Asn-Val-Ser-Thr	
	III-14	113-122 α	Leu-Pro-Ser-Asp-Phe-Thr-Pro-Ala-Val-His	Phe
		(112-121) α	His-Leu-Pro-Ser-Asp-Phe-Thr-Pro-Ala-Val	Phe
	III-15	93-107 β	Asp-Lys-Leu-His-Val-Asp-Pro-Glu-Asn-Lys-Leu-Leu-Gly-Asn-Val	Phe
	III-16	84-98 α	Ser-Asp-Leu-His-Ala-His-Lys-Leu-Arg-Val-Asp-Pro-Val-Asn-Phe	Phe
	III-17	110-125 α	Ala-Ser-His-Leu-Pro-Ser-Asp-Phe-Thr-Pro-Ala-Val-His-Ala-Ser-Leu	Phe
	III-18	101-106 β	Asn-Phe-Lys-Leu-Leu-Gly, Phe-Lys-Leu-Leu-Gly-Asn (102-107 β)	Phe
	III-19	33-34 α	Phe-Leu; Phe-Leu; (128-129 α)	Phe
IV	IV-1	27-29 β	Leu-Gly-Arg; Gly-Arg-Leu (28-30 β); Gly-Arg-Leu-Leu (28-31 β)	
	IV-2	80-83 β	Leu-Lys-Gly-Thr	

Table 1 (continued)

Identification of Peptides from a Bovine Hemoglobin Peptic Hydrolysate Isolated by SE-HPLC Followed by RP-HPLC

Fraction	Peptide No.	Corresp. Fragment	Sequence	Aromatic Amino Acid
IV (cont.)	IV-3	114-120 β	Ala-Arg-Asn-Phe-Gly-Asn-Glu	Phe
	IV-4	84-90 α	Ser-Asp-Leu-His-Ala-His-Lys	
	IV-5	7-13 β	Lys-Ala-Ala-Val-Thr-Ala-Phe	Phe
	IV-6	47-64 α	Asp-Leu-Ser-His-Gly-Ser-Ala-Gln-Val-Lys-Gly-His-Gly-Ala-Lys-Val-Ala-Ala	
	IV-7	6-15 α	Asp-Lys-Gly-Asn-Val-Lys-Ala-Ala-Trp-Gly	Trp
	IV-8	2-20 α	Leu-Ser-Ala-Ala-Asp-Lys-Gly-Asn-Val-Lys-Ala-Ala-Trp-Gly-Lys-Val-Gly-Gly-His	Trp
	IV-9	88-98 α	Ala-His-Lys-Leu-Arg-Val-Asp-Pro-Val-Asn-Phe	Phe
	IV-10	8-29 β	Ala-Ala-Val-Thr-Ala-Phe-Trp-Ser-Lys-Val-His-Val-Asp-Glu-Val-Gly-Gly-Gly-Glu-Ala-Leu-Gly-Arg	Phe, Trp
	IV-11	103-109 β	Lys-Leu-Leu-Gly-Asn-Val-Leu	
	IV-12	99-106 α	Lys-Leu-Leu-Ser-His-Ser-Leu-Leu	
	IV-13	40-51 α	Lys-Thr-Tyr-Phe-Pro-His-Phe-Asp-Leu-Ser-His-Gly	Phe, Tyr
	IV-14	40-56 α	Lys-Thr-Tyr-Phe-Pro-His-Phe-Asp-Leu-Ser-His-Gly-Ser-Ala-Gln-Val-Lys	Phe, Tyr
	IV-15	102-109 β	Phe-Lys-Leu-Leu-Gly-Asn-Val-Leu	Phe
	IV-16	81-97 β	Lys-Gly-The-Phe-Ala-Ala-Leu-Ser-Glu-Leu-His-Cys-Asp-Lys-Leu-His-Val	Phe
V	V-1	137-141 α	Thr-Ser-Lys-Tyr-Arg	Tyr
	V-2	47-61 α	Asp-Leu-Ser-His-Gly-Ser-Ala-Gln-Val-Lys-Gly-His-Gly-Ala-Lys	
	V-3	47-65 α	Asp-Leu-Ser-His-Gly-Ser-Ala-Gln-Val-Lys-Gly-His-Gly-Ala-Lys-Val-Ala-Ala-Ala	
	V-4	12-23 α	Ala-Ala-Trp-Gly-Lys-Val-Gly-Gly-His-Ala-Ala-Glu	Trp
	V-5	2-20 α	Leu-Ser-Ala-Ala-Asp-Lys-Gly-Asn-Val-Lys-Ala-Ala-Trp-Gly-Lys-Val-Gly-Gly-His	Trp
	V-6	130-145 β	Gln-Lys-Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Arg-Tyr-His	Tyr
	V-7	34-46 α	Leu-Ser-Phe-Pro-Thr-Thr-Lys-Thr-Tyr-Phe-Pro-His-Phe	Phe, Tyr

(continued)

Table 1 (continued)

Identification of Peptides from a Bovine Hemoglobin Peptic Hydrolysate Isolated by SE-HPLC Followed by RP-HPLC

Fraction	Peptide No.	Corresp. Fragment	Sequence	Aromatic Amino Acid
V (cont.)	V-8	47-58 α	Asp-Leu-Ser-His-Gly-Ser-Ala-Gln-Val-Lys-Gly-His	
	V-9	32-40 β	Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	Phe, Tyr, Trp
	V-10	31-40 β	Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	Phe, Tyr, Trp
VI	VI-1	137-141 α	Thr-Ser-Lys-Tyr-Arg	Tyr
	VI-2	140-145 β	Leu-Ala-His-Arg-Tyr-His	Tyr
	VI-3	135-141 α	Val-Leu-Thr-Ser-Lys-Tyr-Arg	Tyr
	VI-4	139-145 β	Ala-Leu-Ala-His-Arg-Tyr-His	Tyr
	VI-5	130-145 β	Gln-Lys-Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Arg-Tyr-His	Tyr
	VI-6	131-145 β	Lys-Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Arg-Tyr-His	Tyr
	VI-7	14-23 α	Trp-Gly-Lys-Val-Gly-Gly-His-Ala-Ala-Glu	Trp
	VI-8	129-145 β	Phe-Gln-Lys-Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Arg-Tyr-His	Phe, Tyr
	VI-9	37-46 α	Pro-Thr-Thr-Lys-Thr-Tyr-Phe-Pro-His-Phe	Phe, Tyr
	VI-10	61-70 β	Ala-His-Gly-Lys-Lys-Val-Leu-Asp-Ser-Phe	Phe
	VI-11	61-74 β	Ala-His-Gly-Lys-Lys-Val-Leu-Asp-Ser-Phe-Ser-Asp-Gly-Met	Phe
	VI-12	33-45 α	Phe-Leu-Ser-Phe-Pro-Thr-Thr-Lys-Thr-Tyr-Phe-Pro-His	Phe, Tyr
	VI-13	128-145 β	Phe-Phe-Gln-Lys-Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Arg-Tyr-His	Phe, Tyr
VII	VII-1	135-141 α	Val-Leu-Thr-Ser-Lys-Tyr-Arg	Tyr
	VII-2	138-141 α	Ser-Lys-Tyr-Arg	Tyr
	VII-3	140-145 β	Leu-Ala-His-Arg-Tyr-His	Tyr
	VII-4	13-18 α	Ala-Trp-Gly-Lys-Val-Gly	Trp
	VII-5	12-24 α	Ala-Ala-Trp-Gly-Lys-Val-Gly-Gly-His-Ala-Ala-Glu-Tyr	Tyr, Trp

Table 1 (continued)

Identification of Peptides from a Bovine Hemoglobin Peptic Hydrolysate Isolated by SE-HPLC Followed by RP-HPLC

Fraction	Peptide No.	Corresp. Fragment	Sequence	Aromatic Amino Acid
VII (cont.)	VII-6	7-24 α	Lys-Gly-Asn-Val-Lys-Ala-Ala-Trp-Gly-Lys-Val-Gly-Gly-His-Ala-Ala-Glu-Tyr	Tyr, Trp
	VII-7	24-34 β	Gly-Glu-Ala-Leu-Gly-Arg-Leu-Leu-Val-Val-Tyr	Tyr
	VII-8	130-145 β	Gln-Lys-Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Arg-Tyr-His	Tyr
	VII-9	40-61α	Lys-Thr-Tyr-Phe-Pro-His-Phe-Asp-Leu-Ser-His-Gly-Ser-Ala-Gln-Val-Lys-Gly-His-Gly-Ala-Lys	Tyr, Phe
	VII-10	64-76 β	Lys-Lys-Val-Leu-Asp-Ser-Phe-Ser-Asp-Gly-Met-Lys-His	Phe
	VII-11	64-77 β	Lys-Lys-Val-Leu-Asp-Ser-Phe-Ser-Asp-Gly-Met-Lys-His-Leu	Phe
	VII-12	32-53 α	Met-Phe-Leu-Ser-Phe-Pro-Thr-Thr-Lys-Thr-Tyr-Phe-Pro-His-Phe-Asp-Leu-Ser-His-Gly-Ser-Ala-Gln-Val-Lys-Gly-His	Phe, Tyr
	VII-13	129-145 β	Phe-Gln-Lys-Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Arg-Tyr-His	Phe, Tyr
	VII-14	36-53 α	Phe-Pro-Thr-Thr-Lys-Thr-Tyr-Phe-Pro-His-Phe-Asp-Leu-Ser-His-Gly-Ser-Ala-Gln-Val-Lys-Gly-His	Phe, Tyr

peptic hydrolysate of myoglobin.[8] This may be a result of the interaction between molecules to be separated. In fact, the mobile phase we used in this separation had a lower ionic concentration (7 mM amonium acetate); therefore, the Van der Waals force may have induced these interactions between the molecules and, therefore, became the overriding factor in the resolution of SE-HPLC. This factor may be used as a principal rule in the SE-HPLC separation. Since many peptides of peptic bovine hemoglobin hydrolysate contain aromatic amino acids, the quantitative determination of these peptides, if necessary, was simplified.[26]

It was also observed that some peptides appeared to be broken down into other peptides (F I-9 to F I-1; F II-21 to F II-19, etc). This phenomenon has been previously described for peptic myoglobin hydrolysate.[8] The location of

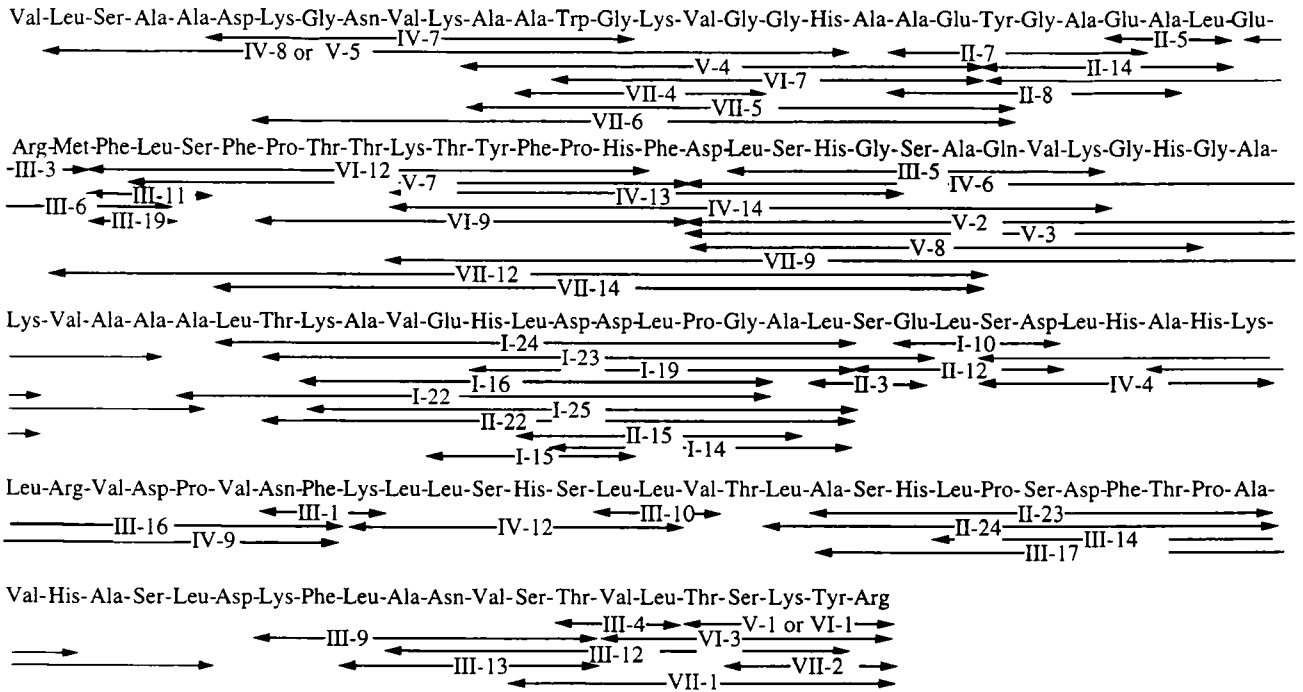

Figure 6. The localization of the peptides isolated from peptic bovine hemoglobin hydrolysate in the α chain of bovine hemoglobin.

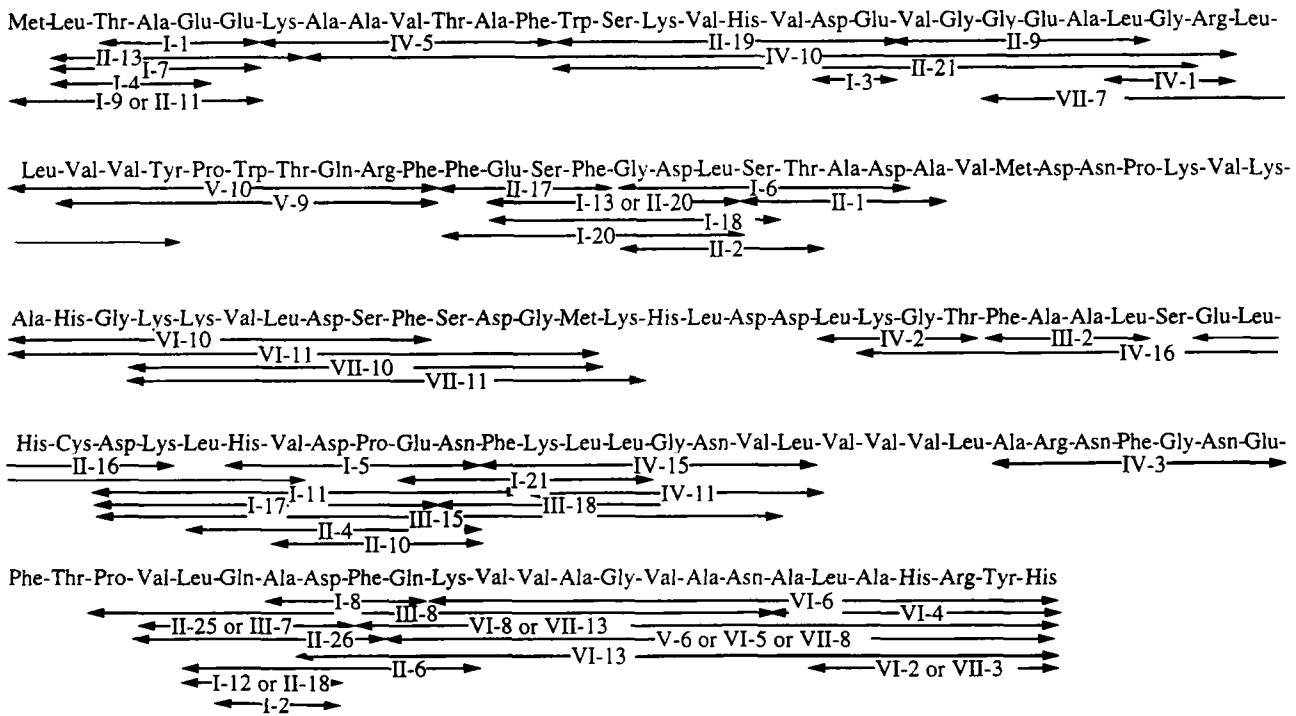

Figure 7. The localization of the peptides isolated from peptic bovine hemoglobin hydrolysate in the β chain of bovine hemoglobin.

these peptides in the α and β chains of bovine hemoglobin confirmed the lower specificity of pepsin. The hydrolysis mechanism of pepsin in an ultrafiltration reactor, however, may be different to that in a batch preparation. We have previously reported that a batch peptic hydrolysis of hemoglobin could constitute a multisubstrate system.[27] The complete evolution of peptides during the hydrolysis should result in a defined order of peptic hydrolysis of hemoglobin, even though pepsin is a rather non-specific enzyme. This means that, for a certain degree of hydrolysis, the composition of the hydrolysate should be defined. This hydrolysis should give rise to the reproducibility of such a hydrolysate. In an ultrafiltration reactor, this hydrolysis mechanism may be modified by the ultrafiltration process. In other words, the variation of the concentration of the substrates relies not only on the hydrolysis process, but also on the ultrafiltration process. However, the reproducible production of such a hydrolysate has been clearly demonstrated.[16]

Mass Spectrometry

In order to confirm the results obtained by amino acid analysis, some peptides were also analysed by mass spectrometry. Figure 8 shows the mass spectrometry profile of peptide F III-16. The molecular weight deduced from $(M+H)^+$ was 1747 daltons. This is in excellent agreement with the MW calculated from the amino acid composition. Regarding the MW of the peptides in the hydrolysate, the peptides MW varied from about 200 to 2000 Daltons (i. e. 2 to 21 amino acids). Moreover, more than 50% of the peptides identified in the bovine hemoglobin hydrolysate were composed of less than 8 amino acids. This may be an advandage for nutritional properties. It is well known that the end products of digestion, i.e., short oligopeptides, are not absorbed in a similar way to that of free amino acids.[28] It has previously been demonstrated that enteric infusions containing small peptides (di- or tri-peptides) were more efficiently assimilated than corresponding free amino acid mixtures and displayed enhanced nutritional value.[29]

Bioactive Peptide Analysis

In relation to the the bioactive peptides, we have already reported some bioactive peptides isolated from this hemoglobin hydrolysate, such as opioid peptides for F V-9 and F V-10[17], bradykinin potentialisating peptide for F III-13[18] and bacteria growth stimulating peptide F II-1[19]. Additionally, it can be seen that peptides F V-1 and F VI-1 have a common sequence with neo-kyotorphin, which was first studied by H. Takagi et al.[30] Its biological

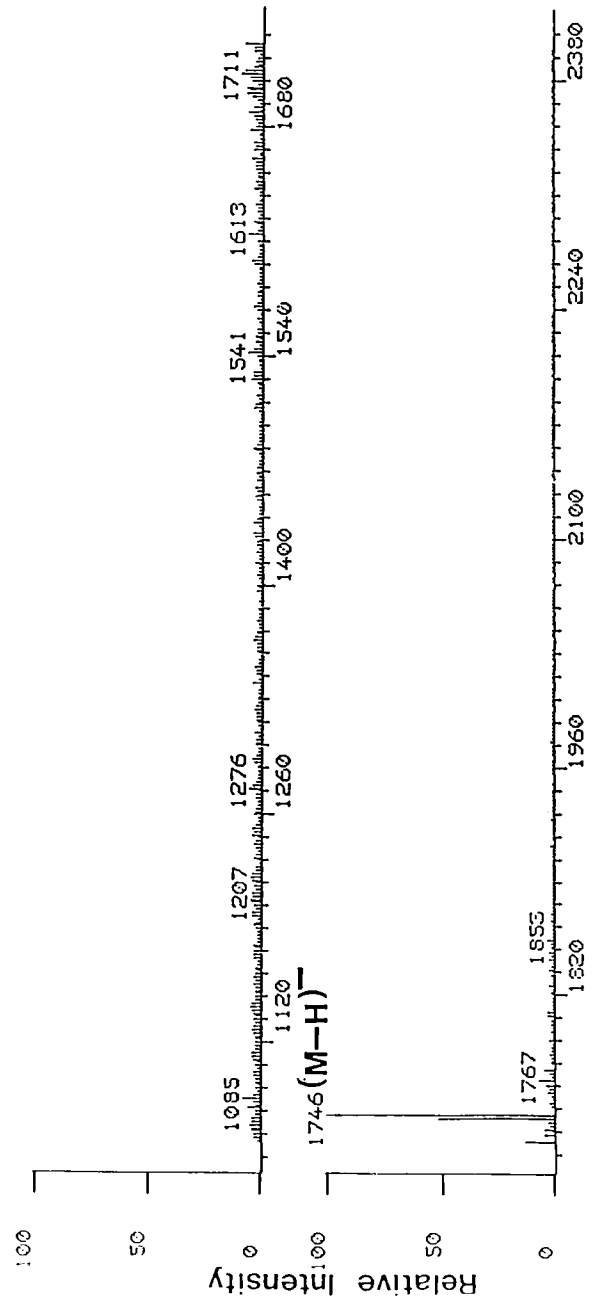

Figure 8. FAB mass spectrometry peptide F III-16. The abundant ion at 1746, designated as a molecular cation $(M+H)^+$, suggested that F III-16 has a molecular weight of 1747.

Table 2

Biological Activity of Some Endogenous Hemoglobin Fragments and Related Peptides Isolated from Peptic Bovine Hemoglobin Hydrolysate

Peptides from Hydrolysate		Endogenous Hemoglobin Fragments			
Peptide	**Localization**	**Position**	**Source**	**Biol. Activity**	**Ref.**
F III-17	110-125 α	110-124 α	Bovine hypo-thalamus	Coronaro-constrictory in vitro	33
F V-5	2-20 α	1-21 α	Bovine hypo-thalamus	Coronaro-constrictory in vitro	33
F V-7	34-46 α	33-46 α	Pig brain	ACTH-releasing in vitro	34
F VI-12	33-45 α				
F VII-1, VI-3	135-141 α	137-141 α	Bovine brain	Analgesic in vivo	30
F VII-2	138-141 α				
F VI-13	128-145 β	133-145 β	Bovine hypo-thalamus	Coronaro-consrictory	33
F VI-8	129-145 β				
F V-6, VI-5	130-145β				

activities such as analgesic, antihibernatic in vivo and ion current regulation in vitro,[30-32] have been investigated. F III-17 has been investigated for its function of potential hydrophobic carrier.[35] It was also observed that some of our peptides have almost identical sequences as those of bioactive peptides of bovine hemoglobin found in vivo (Table 2). Therefore, this indicated that this enzymatic hydrolysate contained many bioactive peptides which can confer an important potential value to this hydrolysate for many applications. Moreover, some of these peptides were the same, or similar to, that found in vivo, signifying that peptic hydrolysis could represent an important physiological hydrolysis model. In other words, these active fragments of hemoglobin found

in vivo may be issued from a peptic-like hydrolysis of hemoglobin during a physiological process. This hydrolysis model has already been proposed for the investigation of the production of certain bioactive peptides.[36]

In conclusion, a peptic bovine hemoglobin hydrolysate, produced at pilot-plant scale, was analyzed fully by SE-HPLC and RP-HPLC. This analysis was completed with second-order derivative spectrometry, amino acid composition analysis, and mass spectrometry, which gave rise to the complete peptidic composition of this hydrolysate. It revealed, at the same time, the presence of some bioactive peptides and some potentially bioactive peptides. These important findings will be of use in potential applications of this enzymatic hydrolysate.

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