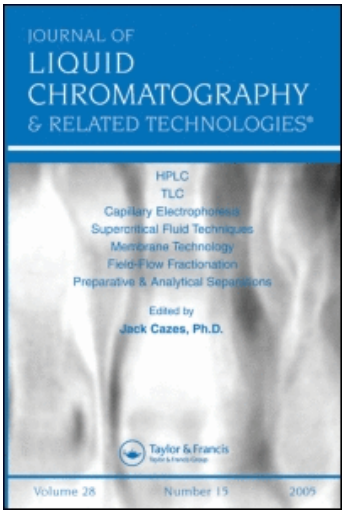

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ANALYTICAL PEPTIDE MAPPING OF A COMPLEX YELLOWFIN TUNA MYOGLOBIN PEPTIC HYDROLYSATE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A method is described for a real identification of any peptides isolated from a complex peptic Yellowfin tuna red muscle myoglobin hydrolysate. A combination of size exclusion and reversed phase high performance liquid chromatography have proved to be a useful strategy for fractionation of such a mixture. This technique enable a large number of pure peptides from the total hydrolysate to be obtained. Peptides were identified and located on the known myoglobin sequence from their amino acid content determined by the Pico-Tag method and a second order derivative spectroscopic method. Location of the peptides allowed us to define effective cut sites of the porcine pepsin. The procedure described in this study will be useful for acquiring a better knowledge of such an hydrolysate.

INTRODUCTION

Proteins which have nutritional qualities as food additives or health qualities as cosmetics additives, are of high valorization potential. In addition to the proteins obtained from conventional sources (terrestrian mammals) which have been largely used these past few years, a new field of molecules issued from non conventional sources has emerged. Thus, recent studies carried out on terrestrian vegetables, seaweeds, and fishes allowed to considere these proteins as good candidates for the replacement of the first ones. For instance, casein hydrolysates, which constitute one of the most classical protein source [1, 2], may be replaced by soybean, wheat or fishes hydrolysates for animal feeding and nutritional therapy [3-6]. Furthermore, it was demonstrated that many edible proteins could lead to biologically active peptides when they are digested by gastrointestinal proteolytic enzymes [7-9]. Thus, it was suggested that food proteins could both constitute a nitrogen source and exert a biological role.

In this paper, tuna fish myoglobin was investigated. Tuna fish is an important source of myoglobin considering the high concentration found in tuna red muscle [10]. In addition, large amounts of tuna red muscle are available. Until now, tuna red muscle valorization was limited to pet food and other feeding manufacturing products. In myoglobin, the protein entity is associated to heminic iron. So, it could constitute not only a potential source of peptides, which have been demonstrated to be the best nitrogen intestinal absorption way [11-12], but also a source of hemin which is the most bioavailable iron form [13-14]. Moreover, as far as biologically active peptides or heminic peptides are concerned, none of them have ever been characterized from a myoglobin enzymatic hydrolysate. Up to now, hydrolyses have only been performed to determine the protein sequence and to understand myoglobin functional properties as oxygen carrier [15-17]. The

purpose of our work was to characterize a myoglobin hydrolysate prior to further studies as to its nutritional or bioactive peptides. A physiological enzyme, pepsin, was employed to hydrolyse yellowfin-tuna myoglobin. Peptides of this hydrolysate were purified by a chromatographic method associating size exclusion HPLC (SE-HPLC) and reversed phase HPLC (RP-HPLC) [18]. The identification and the location of the different peptides in the known myoglobin structure were then realized by aminoacid analysis completed with a second order derivative spectroscopic method to detect aromatic amino acids [19]. Moreover, we compared the specificity of pepsin towards peptidic bonds with that previously described in the literature [20].

MATERIALS AND METHODS

Materials

Red squeletal muscle of yellowfin-tuna was supplied from Paulet Society (Douarnenez, France). Porcine pepsin (EC.3.4.23, 496 Anson unity) was purchased from Sigma. Amino acid analysis reagents were obtained from Pierce. Acetonitrile was of HPLC grade. Other chemicals were of analytical grade. All aqueous HPLC eluants were filtered prior to use on 0.45 µm filters, and degassed with helium during analysis.

All HPLC analysis were performed with a Waters 600E gradient controler-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. Millenium software was used to plot, acquire and treat chromatographic data.

Purification of Yellowfin-tuna Myoglobin

Myoglobin was isolated from the red squeletal muscle of yellowfin-tuna using a method adapted from Suzuki and Sugawara's procedure [21]. The tissue was homogeneized in distilled water (1:1, w:v) in an ice-cold waring blendor. The resulting suspension was centrifuged, (3000 g, 15 min), at 4°C and solid ammonium sulfate was added to the aqueous extract to give 60% saturation. After centrifugation (3000 g, 15 min at 4°C) the pellet, almost free from myoglobin, was discarded. Ammonium sulfate was then added to the supernatant to reach 80% saturation, and the resulting solution was centrifuged (3000 g, 15 min). The whole pellet including myoglobin was dissolved in water and then ultrafiltered and diafiltered (PTGC type membrane, Millipore, 10 KD cut-off) against water. The crude resulting solution, containing myoglobin, but also hemoglobin and other water soluble proteins, was loaded on a D.E.A.E. Sephacel column (32x3cm i.d.) previously equilibrated with 50mM TRIS-HCl buffer (pH 8.6) according to Brown [22]. The flow rate was 60 ml/h.

Enzymatic Hydrolysis

Myoglobin (1g) was dissolved in water (100ml), and the pH was adjusted at 2.0 by HCl. Enzymatic digestion by porcine pepsin (40mg) was performed in batch at 40°C for 3h. The pH was maintained at 2.0 by HCl using a Methrom pHstat. The degree of hydrolysis (DH) was deduced from the volume of HCl consumed during the reaction [23, 24]. In order to stop the reaction, the pH was adjusted to 6.5 with ammonium hydroxide. Insoluble core (proteins partly hydrolysed, large peptides and heme) was removed from the peptic digest by

centrifugation (10000g, 30min). The soluble peptidic mixture was then resolved by size exclusion and reversed phase HPLC.

SE-HPLC

Before hydrolysis, myoglobin purity was checked by SE-HPLC with a TSK G2000 SWG analytical column (600x 7.5mm i.d.). The column was equilibrated with 5mM ammonium acetate/acetic acid buffer, pH 6.0. The proteins were eluted with the same buffer at a flow rate of 0.9 ml/min.

SE-HPLC of peptides generated from the myoglobin peptic digestion was performed using a semi-preparative TSK G 2000 SWG column (600x21.5 mm i.d.). The column was equilibrated with 3mM ammonium acetate/acetic acid buffer, pH 6.0. The peptidic mixture was filtered through 0.22µm filters before being applied to the column and peptides were eluted with the equilibrating buffer at a 6ml/min flow rate. Absorbance was monitored at 215 nm. Peptidic fractions, determined on the basis of UV absorbance, were manually collected and then freeze dried before being applied to the RP-HPLC.

RP-HPLC

Analysis of the peptidic fractions issued from SE-HPLC was carried out on a Delta Pak C18 reversed phase column (300x3,9mm i.d.) previously equilibrated with 10mM ammonium acetate/acetic acid buffer, pH 6.0 (eluant A). Peptidic fractions were reconstituted in eluant A, and filtered through 0.22µm filters before being injected on the column. The analysis was performed with a linear gradient of acetonitrile (eluant B) optimized for each fraction. The flow rate was

1.5ml/min. UV absorbance of the peptides was monitored at 215 nm. The pure peptides obtained from the RP-HPLC column were then freeze dried.

Amino Acid Analysis

Each peptide purified by RP-HPLC was hydrolysed with 6M hydrochloric acid containing 1% phenol, for 24 h at 110°C in sealed glass tubes, using a Waters Pico-Tag station. Amino acids were then analyzed on a Waters RP-Pico-Tag column (150x3.9mm i.d.). Precolumn derivatization of amino acids with phenyl isothiocyanate and HPLC separation of derivatized amino acids was performed according to Bidlingmeyer *et al* [25].The detection wavelength was 254 nm and the flow rate 1ml/min.

Tryptophan could not be detected by this method, because it was destroyed during acid hydrolysis. So, the presence of tryptophan (Trp) and other aromatic amino acids, tyrosine (Tyr) and phenyl alanine (Phe) in peptides was determined by a derivative spectroscopic method [19]. In fact, it has been demonstrated that the second order derivative spectrum allowed to detect aromatic amino acid since their second order derivative spectra exhibited a minimum at 258.5, 283.5, and 289.5 nm for Phe, Tyr and Trp respectively.

RESULTS AND DISCUSSION

Myoglobin was extracted from tuna squeletal red muscle and purified by successive ammonium sulfate fractionation, and ion exchange chromatography. Myoglobin was first eluted with 50mM TRIS-HCl buffer, pH 8.6 (fig. 1). Hemoglobin and other contaminants were subsequently eluted with the same

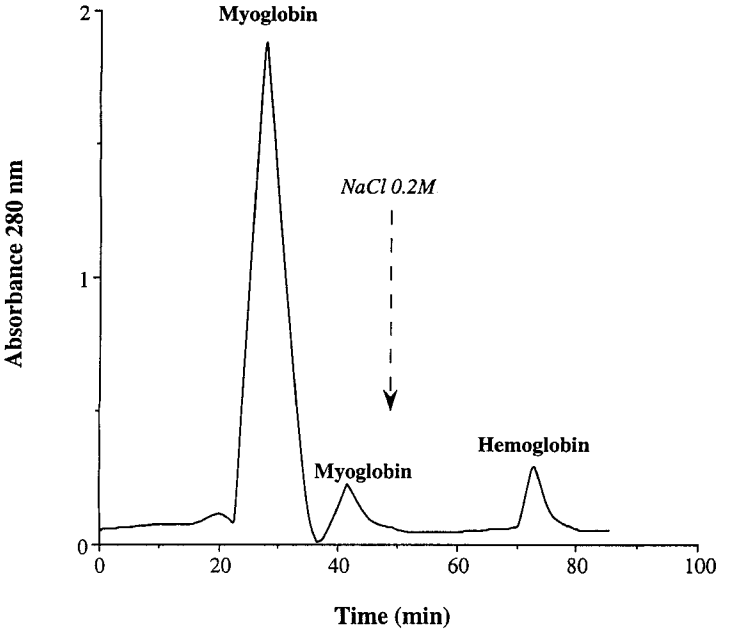

Figure 1. Preparative chromatography on a D.E.A.E. Sephacel column, (320 x 30 mm i.d) of a Yellowfin tuna red muscle extract. Equilibrating buffer: Tris/ HCl 50mM, pH 8.6. Eluting buffers: Tris/ HCl 50mM, pH 8.6 and Tris/HCl 50mM, pH 8.6, NaCl 0.2M. Flow rate: 1.6ml/min.

buffer containing 0.2M NaCl. After extraction and purification, the yield of myoglobin was approximately 56% (w,w).

Yellowfin tuna myoglobin was digested by pepsin for three hours, resulting in a DH of 26% (fig. 2). The generated peptides were resolved by size exclusion HPLC. Fifty injections were performed on the basis of 20mg of total hydrolysate per run. The same elution pattern was obtained for each injection. The chromatogram exhibits seven fractions, numbered I to VII (fig. 3). These fractions were manually collected, pooled, and freeze dried. They were then

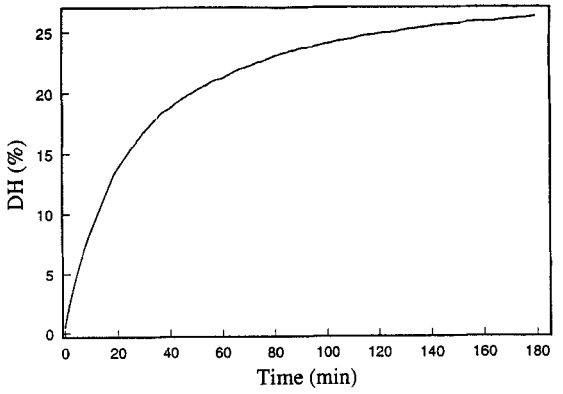

Figure 2. Peptic hydrolysis of Yellowfin tuna myoglobin.
Myoglobin (1g/100ml) was digested by porcine pepsin (40mg/100ml) in a batch at pH 2.0 and 40°C. DH (Degree of Hydrolysis) was measured according to the method of Adler-Nissen [24]

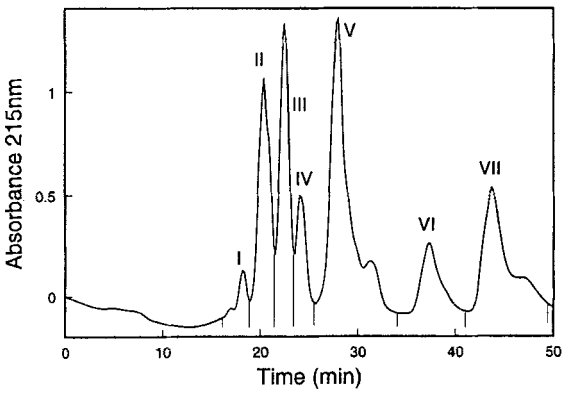

Figure 3. Elution profile of peptic digest of myoglobin on a TSK G2000 SWG semi-preparative column (600x21.5 mm i.d.) equilibrated and eluted with 3.0 mM ammonium acetate / acetic acid buffer, pH 6.0. Injection volume: 100µl (20mg of total hydrolysate). Flow rate: 6.0ml/min.

TABLE 1
Acetonitrile/Ammonium Acetate Buffer Gradient Applied on a RP-HPLC Column, Optimized for Each Fraction. (Eluant A: 10mM Ammonium Acetate/Acetic Acid Buffer, pH 6.0, Eluant B: Acetonitrile).

Fraction	Time (min)	Flow rate (ml/min)	Eluant A %	Eluant B %
I, II	0	1.5	100	0
	6	1.5	94	6
	56	1.5	70	30
	60	1.5	100	0
III, IV	0	1.5	100	0
	56	1.5	72	28
	60	1.5	100	0
V	0	1.5	100	0
	5	1.5	92	8
	55	1.5	74	26
	58	1.5	100	0
VI	0	1.5	100	0
	45	1.5	70	30
	47	1.5	50	50
	50	1.5	100	0
VII	0	1.5	100	0
	5	1.5	90	10
	50	1.5	65	35
	52	1.5	50	50
	55	1.5	100	0

dissolved in 10mM ammonium acetate buffer pH 6.0 to a final peptide concentration of 10mg/ml, and applied to a Deltapak C18 column (1mg per run). In order to improve optimal separation of peptides having largely different hydrophobicities, a specific gradient of acetonitrile/ammonium acetate buffer (10mM, pH6,0) was applied for each fraction (Table 1).

The chromatograms issued from the RP-HPLC separation are presented in figure 4. Four injections of each fraction (I to VII) were performed and individual resulting peaks were collected separately. They have been denoted according to the process of isolation and are represented by Arabic numerals following the Roman numerals. Each peak was checked for homogeneity by RP-HPLC. Thus, eighty-three peptides were collected and freeze dried before amino acid analysis.

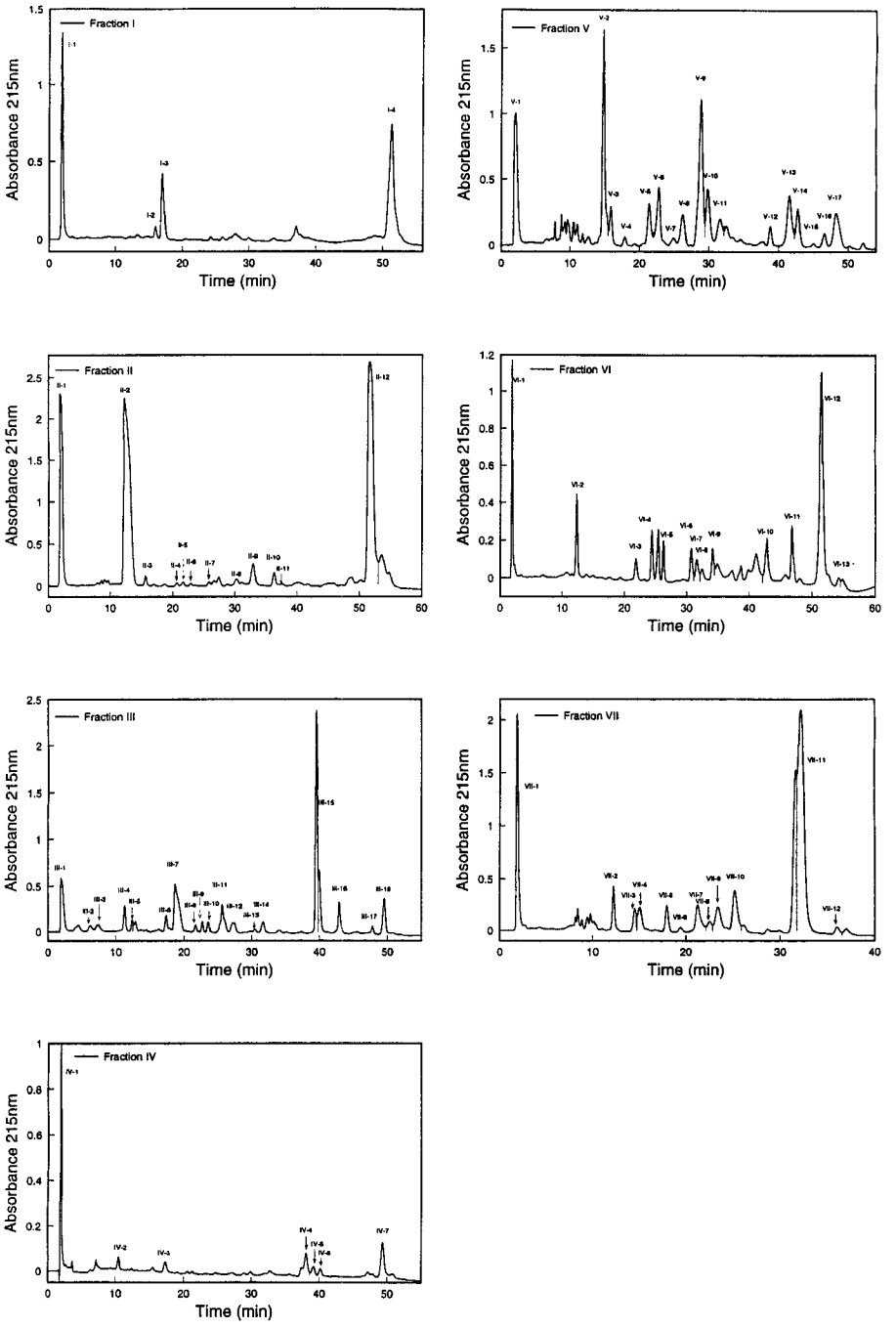

Figure 4. Purification by RP-HPLC on a Deltapak C18 column of the fractions I to VII issus from SE-HPLC. Injection volume: 100µl (1mg peptides). Flow rate: 1.5ml/min.

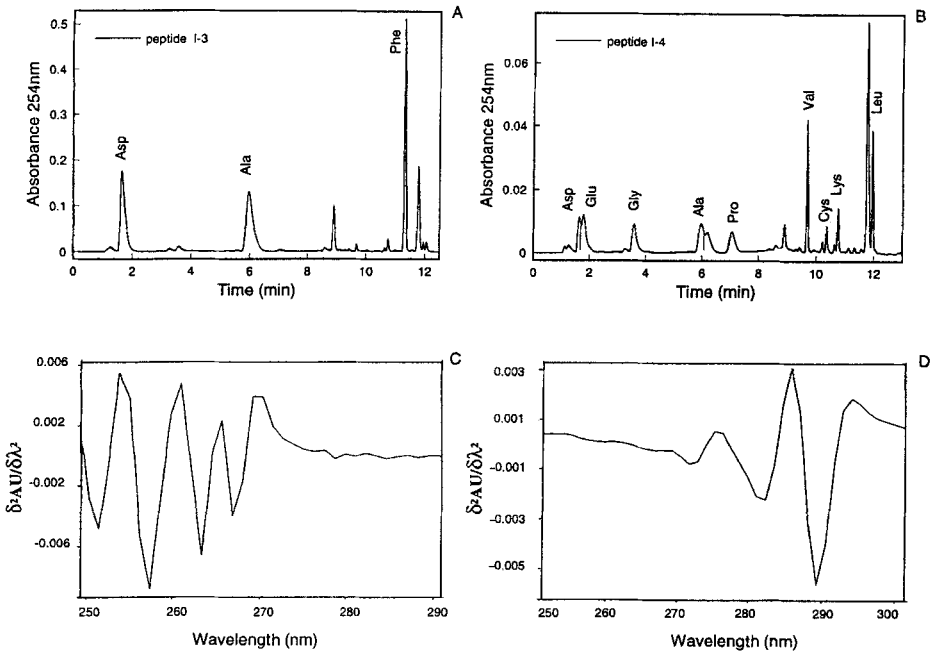

Figure 5. Amino acid analysis ot the peptides I-3 and I-4.
Top: elution profiles of PTC-amino acids for the peptides I-3 (A) and I-4 (B). Peptides amino acids were converted into their PTC derivatives and separed by RP-HPLC on a Pico-Tag column.
Bottom: second order derivative spectra of the peptides I-3 (C) and I-4 (D).

Amino acid analysis of each peptide was performed on the Waters Pico-Tag column after converting amino acids into their PTC (phenylthyocarbamyl) derivatives. This analysis was completed with the second order derivative spectroscopic method [19]. As an example, the amino acids analysis profiles and the second order derivative spectra of the peptides I-3 and I-4 are shown in figure 5. The amino acid analysis allowed us to detect the presence of the amino acids Ala, Asp, Phe in peptide I-3 (fig. 5a) and Leu, Lys, Cys, Gly, Pro, Val, Glu, Ala,

Asp in peptide I-4 (fig. 5b). The presence of Phe in the first peptide was confirmed by the second order derivative spectroscopic method since a minimum was observed at 259 nm (fig. 5c). For the second peptide, a minimum at 289,5 nm revealed the presence of tryptophan, which could not be detected by classical amino acid analysis (fig. 5d). Thus, the identification and location of each peptide purified by RP-HPLC was carried out by the comparison of amino acid composition with the known myoglobin sequence [16] (Table 2).

Peptides molecular weights were deduced from amino acid compositions. So, by peptic hydrolysis of myoglobin, fragments ranging from about 200 to 2000 Daltons (2 to 20 amino acids) were generated. As far as peptides size is considered in SE-HPLC, it can be observed that there was no correlation between retention time and molecular weight. Thus, after TSK gel filtration, most of the largest peptides were eluted in fractions V and VII (fig. 6). Many factors such as hydrophobicity and electric charges can affect the behavior of the peptides during size exclusion chromatography [26]. Owing to this complexity, size exclusion chromatography could be performed only to get a rough separation of peptidic fractions for further RP-HPLC analysis and also to access the reproductibility of the hydrolysate. Taking the complexity of the hydrolysate into account, this two steps separation was particularly effective for the purification of most of the peptides released by myoglobin peptic digestion. In fact, even for the complex peptidic fractions III, V and VI, the high resolution allowed to identify unambiguously the major but also the minor peptides.

More than 50% of the peptides identified in the myoglobin hydrolysate were constituted by less than 7 amino acids residues. This result is in good agreement with the known characteristics of pepsin [27] which is one of the physiological

TABLE 2

Identification of Peptides from an Yellowfin Tuna Red Muscle Myoglobin Peptic Hydrolysate Isolated by SE-HPLC followed by RP-HPLC.

Fractions	Peptide N° from RP-HPLC	Corresponding Myoglobin fragment	Sequence	Aromatic amino acid detected by second order derivative spectrum
I	I-1	112-115	Glu-Lys-Ala-Gly	
	I-2	1-4	Ala-Asp-Phe-Asp	Phe
	I-3	1-3	Ala-Asp-Phe	Phe
	I-4	7-16	Leu-Lys-Cys-Trp-Gly-Pro-Val-Glu-Ala-Asp	Trp
II	II-1	133-134 or 1-2	Ala-Asp	
	II-2 as I-3	1-3	Ala-Asp-Phe	Phe
	II-3	14-17	Glu-Ala-Asp-Tyr	Tyr
	II-4	129-133	Gly-Ile-Ile-Ile-Ala	
	II-5	43-50	Ala-Gly-Ile-Ala-Gln-Ala-Asp	
	II-6	45-52	Ile-Ala-Gln-Ala-Asp	
	II-7	112-117	Glu-Lys-Ala-Gly-Leu-Asp	
	II-8	10-16	Trp-Gly-Pro-Val-Glu-Ala-Asp	Trp
	II-9	10-13	Trp-Gly-Pro-Val	Trp
	II-10	136-146	Glu-Ala-Asn-Tyr-Lys-Glu-Leu-Gly-Phe-Ser-Gly	Tyr
	II-11 as I-4	7-16	Leu-Lys-Cys-Trp-Gly-Pro-Val-Glu-Ala-Asp	Trp
	II-12	7-15	Leu-Lys-Cys-Trp-Gly-Pro-Val-Glu-Ala	Trp
III	III-1	14-16 or 47-49 or 136-138	Glu-Ala-Asp or Gln-Ala-Asp or Glu-Ala-Asn	
	III-2	133-135	Ala-Asp-Leu	
	III-3	15-17 or 137-139	Ala-Asp-Tyr or Ala-Asn-Tyr	Tyr
	III-4	136-140	Glu-Ala-Asn-Tyr-Lys	Tyr
	III-5	46-50	Ala-Gln-Ala-Asp-Ile	
	III-6	5-7	Ala-Val-Leu	
	III-7	4-7	Asp-Ala-Val-Leu	
	III-8	135-139	Leu-Glu-Ala-Asn-Tyr	Tyr
	III-9	130-135	Ile-Ile-Ile-Ala-Asp-Leu	
	III-10	136-143	Glu-Ala-Asn-Tyr-Lys-Glu-Leu-Gly	Tyr
	III-11	102-106	Ile-Ser-Glu-Val-Leu	
	III-12	44-49	Gly-Ile-Ala-Gln-Ala-Asp	
	III-13	14-22	Glu-Ala-Asp-Tyr-Thr-Thr-Met-Gly-Gly	Tyr
	III-14	130-134	Ile-Ile-Ile-Ala-Asp	
	III-15 as II-10	136-146	Glu-Ala-Asn-Tyr-Lys-Glu-Leu-Gly-Phe-Ser-Gly	Tyr,Phe
	III-16	130-135	Ile-Ile-Ile-Ala-Asp-Leu	
	III-17	129-135	Gly-Ile-Ile-Ile-Ala-Asp-Leu	
	III-18	8-16	Lys-Cys-Trp-Gly-Pro-Val-Glu-Ala-Asp	Trp
IV	IV-1	112-116	Glu-Lys-Ala-Gly-Leu	
	IV-2	14-19	Glu-Ala-Asp-Tyr-Thr-Thr	Tyr
	IV-3 as III-7	4-7	Asp-Ala-Val-Leu	
	IV-4 as II-10	136-146	Glu-Ala-Asn-Tyr-Lys-Glu-Leu-Gly-Phe-Ser-Gly	Tyr,Phe
	IV-5	10-15	Trp-Gly-Pro-Val-Glu-Ala	Trp
	IV-6	79-81	Ala-Ile-Leu	
	IV-7	10-16	Trp-Gly-Pro-Val-Glu-Ala-Asp	Trp

(continued)

TABLE 2 (continuation)

Identification of Peptides from an Yellowfin Tuna Red Muscle Myoglobin Peptic Hydrolysate Isolated by SE-HPLC followed by RP-HPLC.

Fractions	Peptide N° from RP-HPLC	Corresponding Myoglobin fragment	Sequence	Aromatic amino acid detected by second order derivative spectrum
V	V-1	145-146	Ser-Gly	
	V-2	17-22	Tyr-Thr-Thr-Met-Gly-Gly	Tyr
	V-3	29-38	Phe-Lys-Glu-His-Pro-Glu-Thr-Gln-Lys-Leu	Phe
	V-4	140-146	Lys-Glu-Leu-Gly-Phe-Ser-Gly	Phe
	V-5	59-64	His-Gly-Ala-Thr-Val-Leu	
	V-6	23-24 or 24-25	Val-Leu or Leu-Val	
	V-7	130-132	Ile-Ile-Ile	
	V-8	54-66	Ala-Ala-Ile-Ser-Ala-His-Gly-Ala-Thr-Val-Leu-Lys-Lys	
	V-9	58-69	Ala-His-Gly-Ala-Thr-Val-Leu-Lys-Lys-Leu-Gly-Glu	
	V-10	139-146	Tyr-Lys-Glu-Leu-Gly-Phe-Ser-Gly	Tyr,Phe
	V-11	129-146	Gly-Ile-Ile-Ile-Ala-Asp-Leu-Glu-Ala-Asn-Tyr-Lys-Glu-Leu-Gly-Phe-Ser-Gly	Tyr,Phe
	V-12	118-128	Ala-Gly-Gly-Gln-Thr-Ala-Leu-Arg-Asn-Val-Met	
	V-13	101-120	Leu-Ile-Ser-Glu-Val-Leu-Val-Lys-Val-Met-His-Glu-Lys-Ala-Gly-Leu-Asp-Ala-Gly-Gly	
	V-14	107-125	Val-Lys-Val-Met-His-Glu-Lys-Ala-Gly-Leu-Asp-Ala-Gly-Gly-Gln-Thr-Ala-Leu-Arg	
	V-15	100-119	Lys-Leu-Ile-Ser-Glu-Val-Leu-Val-Lys-Val-Met-His-Glu-Lys-Ala-Gly-Leu-Asp-Ala-Gly	
	V-16	14-24 or 13-23	(Val)-Glu-Ala-Asp-Tyr-Thr-Thr-Met-Gly-Gly-Leu-(Val)	Tyr
	V-17	1-14	Ala-Asp-Phe-Asp-Ala-Val-Leu-Lys-Cys-Trp-Gly-Pro-Val-Glu	Phe,Trp
VI	VI-1	113-115	Lys-Ala-Gly	
	VI-2	25-27 or 26-28	(Leu)-Thr-Arg-(Leu)	
	VI-3	125-128	Arg-Asn-Val-Met	
	VI-4	125-129	Arg-Asn-Val-Met-Gly	
	VI-5	140-142	Lys-Glu-Leu	
	VI-6	64-69 or 140-143	Leu-Lys-Lys-Leu-Gly-Glu or Lys-Glu-Leu-Gly	
	VI-7	140-144	Lys-Glu-Leu-Gly-Phe	Phe
	VI-8	68-70	Gly-Glu-leu	
	VI-9	63-71	Val-Leu-Lys-Lys-Leu-Gly-Glu-Leu-Leu	
	VI-10	143-146	Gly-Phe-Ser-Gly	Phe
	VI-11	107-116	Val-Lys-Val-Met-His-Glu-Lys-Ala-Gly-Leu	
	VI-12	33-44	Pro-Glu-Thr-Gln-Lys-Leu-Phe-Pro-Lys-Phe-Ala-Gly	Phe
	VI-13	2-14	Asp-Phe-Asp-Ala-Val-Leu-Lys-Cys-Trp-Gly-Pro-Val-Glu	Phe,Trp
VII	VII-1	28-41	Leu-Phe-Lys-Glu-His-Pro-Glu-Thr-Gln-Lys-Leu-Phe-Pro-Lys	Phe
	VII-2	74-87	Lys-Gly-Ser-His-Ala-Ala-Ile-Leu-Lys-Pro-Leu-Ala-Asn-Ser	
	VII-3	72-79	Lys-Ala-Lys-Gly-Ser-His-Ala-Ala	
	VII-4	66-71	Lys-Leu-Gly-Glu-Leu-Leu	
	VII-5	28-41 or 36-39	Leu-Phe-Lys-Glu or Gln-Lys-Leu-Phe	Phe
	VII-6	84-95	Leu-Ala-Asn-Ser-His-Ala-Thr-Lys-His-Lys-Ile-Pro	
	VII-7	88-104	His-Ala-Thr-Lys-His-Lys-Ile-Pro-Ile-Asn-Asn-Phe-Lys-Leu-Ile-Ser-Glu	Phe
	VII-8	80-85	Ile-Leu-Lys-Pro-Leu-Ala	
	VII-9	36-41	Gln-Lys-Leu-Phe-Pro-Lys	Phe
	VII-10	39-44	Phe-Pro-Lys-Phe-Ala-Gly	Phe
	VII-11	29-44	Phe-Lys-Glu-His-Pro-Glu-Thr-Gln-Lys-Leu-Phe-Pro-Lys-Phe-Ala-Gly	Phe
	VII-12	34-46	Glu-Thr-Gln-Lys-Leu-Phe-Pro-Lys-Phe-Ala-Gly-Ile-ala	Phe

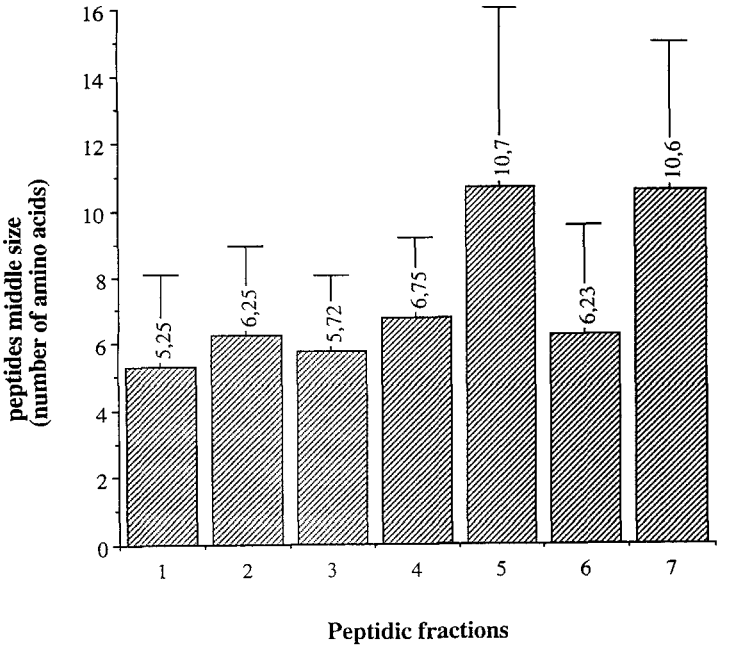

Figure 6. Peptides middle size distribution of each peptidic fraction obtained by SE-HPLC (in amino acids).

proteases exhibiting the lowest specificity. It could be observed that some peptides seemed to be broken down into other peptides, e.g. peptide V-11 is partly converted into III-17 and II-10 and peptide III-6 into III-7. The location of these peptides in the myoglobin structure allowed us to specify the pepsin cut sites towards this protein in our hydrolysis conditions. Effective pepsin cut sites on myoglobin and theoretical pepsin cut sites found in the literature were indicated in fig. 7. If some cleavage sites previously described were confirmed, new sites appeared in our study. This corroborated the weak specificity of pepsine and illustrated how it could be difficult to predict peptidic composition of such an hydrolysate.

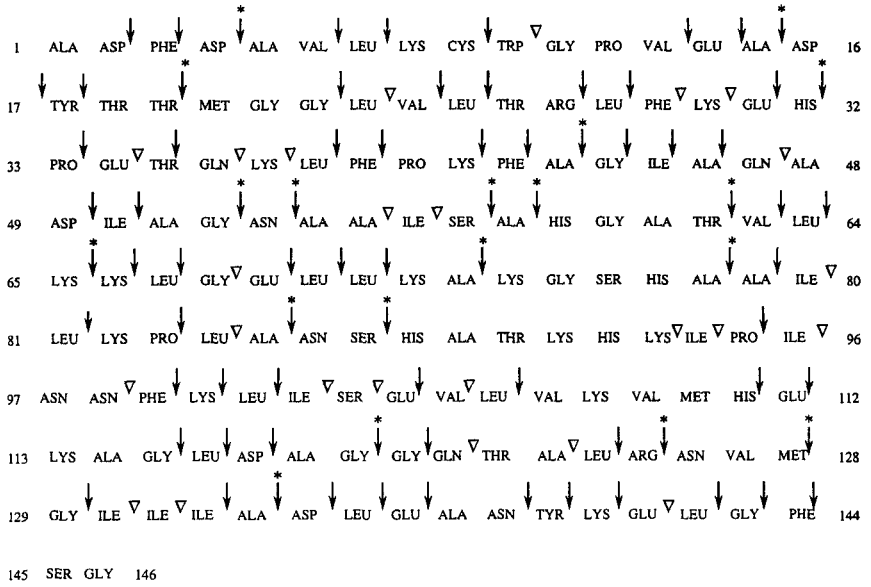

Figure 7. Yellowfin tuna myoglobin sequence determined by Rice *et al.* [16]
↓: pepsin cut sites observed during myoglobin hydrolysis in accordance with those described in the literature.
↓: pepsin cut sites observed during myoglobin hydrolysis and not described in the literature.
∇: pepsin cut sites described in the literature and not observed during myoglobin hydrolysis.

It is well known that the end products of digestion, i.e. short oligopeptides and amino acids are not absorbed in a similar mode [28]. It was previously demonstrated that enteral infusions containing small peptides (di or tri peptides) were more efficiently assimilated than corresponding free amino acid mixture and displayed better nutritional value [29]. In other respects, nutritional studies have been carried out to provide a better understanding of the mechanisms involved in iron absorption. Different pathways could be concerned depending on

whether the iron is heme-iron or free-iron. It has been observed in previous studies that hemoglobin-iron and myoglobin-iron [14] were absorbed more efficiently than inorganic iron. Furthermore, it was established that the iron could enter intestinal cells as heme [14]. Moreover, the importance of the protein was suggested since it has been observed that the degraded globin increased heme absorption probably by binding the coordinating bonds of heme and preventing its polymerization [14]. More recently, it was suggested that small peptides could facilitate iron absorption into the intestinal mucosal cells [30].

In this work, we have demonstrated that peptic hydrolysis of yellowfin tuna myoglobin generated mostly small peptides. Eighty-three peptides have been purified and identified in the complex peptidic mixture. Most of them consisted of less than seven amino acids. It should be interesting to study the nutritional importance of this hydrolysate both for the nitrogen absorption as small peptides and iron absorption as iron-heme or peptide-iron-heme. Moreover, it would be a real chalenge to investigate the potential biological activity of these peptides. In fact, it has been established that peptic digestion of many proteins, especially of hemoglobin [31, 32], results in the production of subtances that were found to have opioïd-like activity.

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