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Obtaining antimicrobial peptides by controlled peptic hydrolysis of bovine hemoglobin

Estelle Yaba Adje[a], Rafik Balti[a,b,*], Mostafa Kouach[c], Pascal Dhulster[a], Didier Guillochon[a], Naïma Nedjar-Arroume[a]

[a] Laboratoire de Procédés Biologiques, Génie Enzymatique et Microbien, IUT « A » Polytech'Lille -Lille I, BP 179, 59653 Villeneuve d'Ascq Cedex, France
[b] Laboratoire de Génie Enzymatique et de Microbiologie - Ecole Nationale d'Ingénieurs de Sfax, B.P "1173" 3038 Sfax, Tunisia
[c] Laboratoire d'Application de Spectrométrie de Masse, Service Commun de Physicochimie, Faculté de Médecine H. Warembourg, Pôle Recherche, Place de Verdun, Lille II, France

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ABSTRACT

Under standard conditions, the peptides and specially the active peptides were obtained from either the denatured hemoglobin that all structures are completely modified or either the native hemoglobin where all structures are intact. In these conditions, antibacterial peptides were isolated from a very complex peptidic hydrolysate which contains more than one hundred peptides having various sizes and characteristics, involving a complex purification process. The new hydrolysis conditions were obtained by using 40% methanol, 30% ethanol, 20% propanol or 10% butanol. These conditions, where only the secondary structure of hemoglobin retains intact, were followed in order to enrich the hydrolyzed hemoglobin by active peptides or obtain new antibacterial peptides. In these controlled peptic hydrolysis of hemoglobin, a selective and restrictive hydrolysate contained only 29 peptides was obtained. 26 peptides have an antibacterial activity against *Micrococcus luteus*, *Listeria innocua*, and *Escherichia coli* with MIC from 187.1 to 1 μM. Among these peptides, 13 new antibacterial peptides are obtained only in these new hydrolysis conditions.

1. Introduction

Antimicrobial peptides are usually made up of between 12 and 50 residues, and while they differ widely in sequence and structure, they do share some common features in that they are cationic, 50% of their amino acids are hydrophobic, and they are generally amphipathic [1–3]. Although being the most active [4], low molecular weight peptide-based compounds, mostly with unknown secondary structure, are known as possessing systemic toxicity and high production costs [5,6]. In the search for such new antimicrobial peptides, the group of α-helical peptides, hydrophobic and cationic has attracted increasing research and clinical interest during the last decade [1,7]. Discovery of hydrolysis condition for obtaining peptides with more important molecular weight, able to adopt α helix structure in contact with the bacterial membrane, is of considerable interest. Also, the microenvironment of the lipid bilayer has been shown to have a stabilizing effect on the α-helical structure of peptides [8]. Realization of a limited hydrolysis can be an alternative to obtain these interest peptides. Characteristics of peptides released from protein hydrolysis are strongly linked to the mechanism occurring. Our study is focused on the research of these new hydrolysis conditions allowing to the obtaining of these peptides from peptic hydrolysis of bovine hemoglobin.

Hemoglobin hydrolysis involved two main mechanisms depending on his structure. In previous work [4,9–11], many authors have report that from early stage of peptic hemoglobin hydrolysis, antibacterial peptides were isolated from a very complex peptidic hydrolysate which contains more than one hundred peptides having various sizes and characteristics, involving a complex purification process. They were obtained when hemoglobin hydrolysis was carried out in denatured state in the presence of urea 6–8 M.

In fact, when hemoglobin is in denatured state, the hydrolysis process leads to formation and successive cleavage of intermediate peptides more or less stable: "zipper mechanism" [12,13]. Hydrolysate resulting from this hydrolysis contained peptides with various sizes and various physical characteristics. A dissociation of the tetrameric structure of hemoglobin is observed in this denatured state. Pepsin cleavage sites were more accessible and hemoglobin hydrolysis was uncontrolled and unlimited. Hemoglobin was rapidly converted into intermediate or final peptides. The intermediate peptides are peptides which the

* Corresponding author at: Laboratoire de Génie Enzymatique et de Microbiologie - Ecole Nationale d'Ingénieurs de Sfax, B.P "1173" 3038 Sfax, Tunisia. Tel.: +33 3 28 76 73 90; fax: +33 3 28 76 73 81.
E-mail address: rafikbalti1981@gmail.com (R. Balti).

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concentration increases in the course of the hydrolysis before decreasing and completely disappearing. Generally, these peptides were characterized were with molecular mass more than 2000 Da, and presented structural characteristic which confer the antibacterial activity.

While, in native state, hemoglobin was hydrolyzed in small size peptides and this hydrolysis mechanism was called the "one by one" mechanism [12]. Thus, 'one-by-one' and 'zipper' mechanisms are respectively for peptic hydrolysis of native and denatured hemoglobin.

Realization of new hydrolysis conditions, where hemoglobin was in structural state different of denatured and native state, can permit to enrich the hemoglobin hydrolysate with active peptides and to obtain new antibacterial peptides.

An alternative which can allow structural modification of bovine hemoglobin was the use of alcohol. Therefore, several studies are show that protein hydrolysis in the presence of alcohol can permitted to induce a limited hydrolysis leading to peptidic population different from those obtain in the absence of alcohols [14–16]. Indeed, alcohols are commonly used to induce new structures or a partially unfolded state in proteins [17–19]. These changes are due to the disturbance of the hydrophobic interaction which would involve a change of conformation [20]. Moreover, alcohols with the long side chains have a stronger inhibitory effect on hydrolysis by enzyme than those with short side chains [21]. The increase in the hydrocarbon chain involves an increase in the denaturing ability of alcohol [22]

In this paper, our objective was to develop new approaches to facilitate production of only intermediate peptides after peptic hydrolysis of bovine hemoglobin. Alcohols were used to induce structural change of hemoglobin as previously described and to realize a limited hydrolysis. pH being in relationship with hemoglobin structure [23], to avoid his effect, pH 5.5 was used. We reported methanol, ethanol, propanol and butanol effects on the structural changes of bovine hemoglobin and identification of novel antimicrobial peptides from limited peptic hemoglobin hydrolysis. Peptic hydrolysis was carried out in the presence of the previous alcohols at 23 °C and pH 5.5, as pepsin remains active at this pH [24,25] and in the presence of some concentration of alcohols [26]. UV–vis spectrophotometer, spectrofluorometer and circular dichroism were used to characterize structural changes of hemoglobin.

2. Methods

2.1. Materials

All common chemicals and solvents were of analytical grade from commercial sources. Bovine hemoglobin and pig pepsin (3260 units/mg protein) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Acetonitrile was of HPLC grade. Water was obtained from a Millipore Milli-Q system; the resistance was about MΩ. All aqueous HPLC eluents were bubbled with Waters in Line Degasser.

2.2. Peptic hydrolysis of bovine hemoglobin

Bovine hemoglobin solution (100 ml) at 1% (w/v) was digested by pig pepsin (EC 3.4.23.1) at 23 °C in 0.1 M sodium acetate buffer, pH 5.5, in homogeneous solution in the absence or in the presence of methanol (1, 10, 20, 30 and 40% (v/v)), ethanol or propanol (1, 10, 20 and 30% (v/v)), butanol (1, 2.5, 5 and 10%) and urea 6 M. The enzymatic hydrolysis was stopped by addition of disodium tetraborate (0.32 M, pH 12.7) up to a final pH of 10.0, leading to the enzyme inactivation. To isolate active intermediate peptides, enzymatic hydrolysis of hemoglobin was performed at 1% degree of hydrolysis (DH) according to the trinitrobenzene sulphonate method of Nielsen [27]. Samples were stored at −20 °C before further analysis.

Table 1
Presence or absence of intermediate peptide released during the course of peptic hydrolysis of hemoglobin in absence or in presence of alcohols with retention time from 35 to 40 min according the RP-HPLC chromatograms at 215 nm (chromatograms was not shown).

	Alcohol concentration (% v/v)	Hydrolysis time (min)						
		2.5	20	30	60	120	180	1444
Methanol	1	–	–	–	–	–	–	–
	10	–	–	–	–	–	–	–
	20	–	–	–	–	–	–	–
	30	–	–	–	–	–	–	–
	40	++	++	++	++	++	++	–
Ethanol	1	–	–	–	–	–	–	–
	10	–	–	–	–	–	–	–
	20	–	–	–	–	–	–	–
	30	++	++	++	++	++	++	–
Propanol	1	–	–	–	–	–	–	–
	10	–	–	–	–	–	–	–
	20	++	++	++	++	++	++	–
	30	+	+	+	+	+	+	–
Butanol	1	–	–	–	–	–	–	–
	2.5	–	–	–	–	–	–	–
	5	–	–	–	–	–	–	–
	10	++	++	++	++	++	++	–
Absence of alcohol	0	–	–	–	–	–	–	–

−: Absence of intermediate peptides; +: presence of intermediate peptides with a lower absorbance; ++: presence of intermediate peptides with higher absorbance. The selected alcohols for further analyses were labeled by the gray color.

2.3. Reverse-phase high-pressure liquid chromatography (RP-HPLC)

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.

All of the chromatographic processes were performed on a Vydac C_4 column (250 mm × 10 mm). The mobile phase was water/trifluoroacetic acid (1000:0.1, v/v) as eluent A and acetonitrile/water/trifluoroacetic acid (600:400:0.1, v/v) as eluent B. The flow rate was 0.6 ml/min. Samples were filtered through 0.22 mm filters and then injected. The gradient applied was 0–67% (v/v) B over 30 min, then 67–87% (v/v) B over 35 min. Online UV absorbance scans were performed between 200 and 300 nm at a rate of 1 spectrum/s with a resolution of 1.2 nm. Chromatographic analyses were completed with Millennium software [28].

2.4. Mass spectrometry analysis

The molecular mass and peptide sequencing were done on positive ion mode using electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (MS/MS), respectively. ESI mass spectrometry was performed using a triple quadrupole instrument Applied Biosystems API 3000 (PE Sciex, Toronto, Canada) equipped with an electrospray ion source. The system is controlled by the Analyst Software 1.4, allowing the control of the spectrometer, the analysis and the processing data. Interpretations of MS/MS spectra were made with Bioanalyst software. The freeze-dried samples from RP-HPLC were dissolved in acetoni-