Antibacterial activity of a pepsin-derived bovine hemoglobin fragment

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Received 22 November 2000; revised 20 January 2001; accepted 25 January 2001

First published online 2 February 2001

Edited by Pierre Jolles

Abstract Peptic digestion of bovine hemoglobin yields a fragment with antibacterial activity. This peptide was purified to homogeneity by a two-step procedure including anion exchange chromatography and preparative reversed-phase HPLC. Mass determination and fragmentation indicated that this peptide corresponded to the 1–23 fragment of the α chain of hemoglobin. The minimum inhibitory concentration and mode of action of this peptide towards *Micrococcus luteus* strain A270 were determined. Hemolytic assay, interaction with liposomes, and study of its structure in solution were also performed.

Key words: Antimicrobial peptide; Bovine hemoglobin; Pepsin

1. Introduction

For many years, protein hydrolysates derived from different sources of agricultural proteins (soya, casein, wheat, or fish) have been used as food ingredients [1]. Additional applications such as nutritional therapy [2], stimulating effects on fermentation [3], immunostimulating properties [4], or biological activities such as antimicrobial activities have also been investigated. Antibacterial activities of hydrolysates derived from bovine [5] or human [6] lactoferrin, bovine α-casein [7], bovine α-lactalbumin [8], or bovine chromaffin granules [9] have been described.

Bovine hemoglobin has also been used as a substrate to obtain organoleptic and bioactive peptides by enzymatic hydrolysis such as a bitter peptide [10] or a bacterial growth-stimulating peptide [11]. Hemoglobin has also been described as a source of endogenous bioactive peptides [12]. Recently, the antibacterial activity of a peptide was found in the gut of the tick *Boophilus microplus* [13]. This peptide, corresponding to the 33–61 fragment of the α chain of bovine hemoglobin, was most potent against *Micrococcus luteus* A270, a bacterial strain commonly used as a sensitive strain for the detection of antibiotic substances [14].

In our laboratory, different peptides have already been obtained from peptic hydrolysis of bovine hemoglobin. Thus, a bradykinin-potentiating peptide [15,16], opioid peptides [17], or amphiphilic peptides used in photochemotherapy for their ability to solubilize and carry protoporphyrin IX in solution [18] have been described for medical applications to deliver hydrophobic photosensitizers to tumor tissues [19].

Among these amphiphilic peptides, the 1–23 fragment of the α chain of bovine hemoglobin, which is an efficient carrier of the porphyrin, is easily and rapidly purified in large quantities [20]. This α 1–23 fragment was tested for its antibacterial activity towards *M. luteus* A270. The present paper is the first report on the antibacterial activity of a peptide isolated from a peptic bovine hemoglobin hydrolysate.

2. Materials and methods

2.1. Preparation and purification of the α 1–23 peptide

Bovine hemoglobin hydrolysate was obtained as a powder on a pilot-plant scale by pepsin proteolysis in an ultrafiltration reactor followed by decolorization (removing of heme) with MgO, desalting and atomization, as previously described [21].

The α 1–23 peptide was purified by the method previously proposed [20] and slightly modified.

Anion exchange chromatography was performed using a 380 ml Q-Sepharose Fast Flow gel (Bio Process Medium) in a XK 50 column (Pharmacia; 300×50 mm). 475 mg of peptic hydrolysate dissolved in 10 ml of 50 mM ethanolamine–HCl buffer, pH 10.5, was applied to the column previously equilibrated with the same buffer. After a 300 ml wash, elution was performed with the same buffer containing different NaCl concentrations: 600 ml buffer (25 mM NaCl), 1350 ml buffer (45 mM NaCl), and then 300 ml buffer (1 M NaCl) to clean and regenerate the column. The flow rate was 5 ml/min. Detection of peptides was done at 280 nm. Five runs in a row were performed and fractions corresponding to the different peaks were collected and kept at 4°C. Fractions containing the α 1–23 peptide were then pooled, desalted by a pilot-plant electrodialysis apparatus as previously described [20], and lyophilized. The peptide powder was then dissolved in 2 ml distilled water and peptides were separated by preparative reversed-phase (RP) HPLC. The apparatus was a Waters 600E pump system controller connected to a LC spectrophotometer (Waters) set at 226 nm and separation was performed using a preparative Waters C18 column (300×19 mm). The mobile phase was water/trifluoroacetic acid (1000:1, v/v) as eluent A and acetonitrile/water/trifluoroacetic acid (600:400:1, v/v) as eluent B. The gradient applied was linear from 3 to 25% B over 10 min and from 25 to 30% B over 80 min. The flow rate was 13.6 ml/min and two injections of 1 ml each were performed. Each fraction was manually collected and lyophilized. Purity was checked by mass spectrometry and analytical RP-HPLC.

2.2. Purity of the peptide

Mass determination was performed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) with a Finnigan Mat Vision 2000 time-of-flight mass spectrometer equipped with a viewing

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0014-5793/01/$20.00
PII: S0014-5793(01)02171-8

unit. All data were acquired at 500 MHz sampling frequency in reflectron mode with external and/or internal calibration and also in linear mode. The nitrogen laser spot size was 70 μm and the laser energy was adjusted close to threshold. The following matrix was used: a mixture of 2,5-dihydroxybenzoic acid (12 g/l) and 2-hydroxy-5-methoxybenzoic acid (12 g/l) (9:1, v/v) was prepared with acetonitrile/water (1:2, v/v) as solvent. A 1 μl portion of the matrix solution was mixed with 0.5 μl of sample solution on a stainless steel target, dried in a gentle stream of air and loaded into the mass spectrometer. Mass spectra were obtained preferably from the crystalline rim of the dried sample. Usually, 15–30 spectra were averaged in linear mode and 20–40 in reflectron mode. Linear configuration was used applying 30 keV ion energy for the reflectron time-of-flight analyzer, 5 keV ion energy and 20 keV post-acceleration to a discrete dynode secondary electron multiplier in positive ion mode.

The analytical RP-HPLC 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 the chromatographic processes were performed with an analytical Vydac C4 column (250×4.6 mm). The mobile phase was water/trifluoroacetic acid (1000:1, v/v) as eluent A and acetonitrile/water/trifluoroacetic acid (600:400:1, v/v) as eluent B. The flow rate was 1 ml/min. Samples were filtered through 0.22 μm 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. On-line UV absorbance scans were performed between 200 and 300 nm at a rate of one spectrum per second with a resolution of 1.2 nm. Chromatographic analyses were completed with Millennium software.

2.3. Bacterial strain, culture media and antimicrobial assays

M. luteus A270 was used as the test microorganism for determination of antibacterial activity. The strain was conserved at −24°C in glycerol-containing nutrient broth and was subcultured twice in Muller–Hinton broth (Biokar Diagnostics) under agitation (60 rpm) at 30°C before use.

Antibacterial activity of the peptide was evaluated by a liquid growth inhibition assay. For determination of the minimum inhibitory concentration (MIC), the strain was cultured in a series of tubes containing 1 ml of Muller–Hinton broth with various concentrations of α 1–23 peptide (1.5 μg/ml–1.5 mg/ml). A standard inoculum of 16 h culture cells was added to each tube at a final concentration of 1×10^6 CFU/ml. The MIC was taken as the lowest concentration of peptide that caused 100% growth inhibition after 24 h of incubation at 30°C under agitation at 60 rpm. For bactericidal activity assay, a suspension of *M. luteus* strain A270 corresponding to the MIC tube after 24 h of incubation was serially 10-fold diluted in tryptone salt solution and plated onto Petri plate count agar (15 g/l) for CFU determination. Incubation of plates was for 72 h at 30°C.

2.4. Hemolytic assay

The hemolytic activity of the peptide was determined using bovine erythrocytes by methods previously proposed [9,22], slightly modified. After isolation of the erythrocytes by centrifugation (1000×*g* for 5 min), they were washed three times with 10 mM sodium phosphate, pH 7.5, containing 0.9% NaCl (NaCl/P_i). The cell concentration stock suspension was adjusted to 1×10^9/ml. The cell suspension (12.5 μl), varying amounts of peptide stock solution (10 mg/ml in NaCl/P_i), and NaCl/P_i were pipetted into Eppendorf tubes to give a final volume of 50 μl. The suspension containing 2.5×10^8 cells/ml was then incubated for 40 min at 37°C. After centrifugation (1000×*g* for 5 min), 30 μl of supernatant was diluted in 500 μl water. The absorbance of the diluted solution was measured at 420 nm. The absorbances obtained after treating erythrocytes with only NaCl/P_i and 0.2% SDS were taken as 0% and 100%, respectively.

2.5. Liposome preparation and liposome content leakage

Lipid films were obtained by evaporating a solution of lipid in chloroform (phosphatidyl ethanolamine (1 mg), phosphatidyl glycerol (0.3 mg) and cardiolipin (0.15 mg)). The lipids were then hydrated in 1 ml Tris–HCl buffer (20 mM, pH 7.0) containing carboxyfluorescein (100 mM) by vortex-mixing for 10 min. The solution was readjusted to pH 7.0 with NaOH (1 M). The suspension was freeze-thawed for five cycles and then successively extruded five times though a polycarbonate filter (0.1 μm pore size). Then, carboxyfluorescein-containing liposomes were subjected to gel filtration (Sephadex G75, 200×10 mm) ion Tris–HCl buffer. Two peaks were collected: the first one corresponded to the liposomes and the second one to the free carboxyfluorescein. The lipid concentration was determined by phosphorus analysis [23].

Tris–HCl buffer (20 mM, pH 7.0) was added to the liposome suspension so that the final concentration of lipids was 2.5 mM. Peptide solutions (25, 37.5, and 50 μM) were added to the diluted liposomal suspension (3 ml). Thus, lipid/peptide ratios were 100:1, 67:1 and 50:1, respectively. Carboxyfluorescein was excited at 470 nm and the emission was monitored between 450 and 700 nm with a fluorescence spectrophotometer (Perkin Elmer LS 50B). The percentage of dye leakage (PDL) induced by the peptide was evaluated using the equation $PDL=[(F-F_0)/(F_t-F_0)]\times100$, where F is the fluorescence intensity after the addition of the peptide, and F_0 and F_t are the fluorescence intensity without the peptide before and after addition of Triton X-100 (2 g/l), respectively. Triton X-100 was used to release completely the dye from the liposomes (100% dye leakage).

2.6. Structure of the peptide in solution

The aggregation state of the peptide was studied by UV spectrophotometry (280 nm). The absorbance of various solutions of the peptide (0–300 μM) in ammonium acetate buffer (10 mM, pH 7) was recorded. Then, separation of the peptide was performed by exclusion chromatography. The column used, Superdexpeptide HR 10/30 (Pharmacia; 300×10 mm), was calibrated with: serum albumin (67 000 Da), A chain of bovine insulin (2531 Da), angiotensin II (1046 Da), β-NAD (663 Da), and glutathione (307 Da). 50 μl of peptide at a concentration of 10 mg/ml was applied. The buffer was ammonium acetate buffer (10 mM, pH 7) and the flow rate was 0.5 ml/min.

Infrared spectra were realized with a Brücker IFS-48 spectrometer, after drying the solution of peptides on silica chips. A spectrum represents the accumulation of 200 recordings with a resolution of 4 cm^{-1} in the range 4000–400 cm^{-1}. Spectra in Fourier transform were obtained by means of the standard Brücker software and interpreted according to previous papers [24,25]. Three concentrations of α 1–23 peptide were tested, 1.25, 2.5, and 5% (w/v), in ammonium acetate buffer (20 mM, pH 7.0).

3. Results and discussion

3.1. Isolation of the peptide

Chromatography used in this study was developed in two steps to obtain a sufficient quantity of the α 1–23 peptide in order to study its biological activity and its structure in solution.

After the first step of bovine hemoglobin hydrolysate fractionation by anion exchange chromatography using a Q-Sepharose Fast Flow gel, 177 mg of peptide fraction was obtained from 2375 mg of the initial peptic hydrolysate. After the second step of purification by preparative reverse-phase HPLC, 37 mg of peptide powder was obtained.

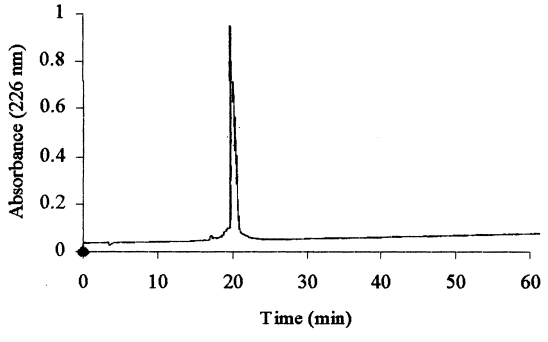

Fig. 1. Analytical RP-HPLC demonstrating the purity of the α 1–23 peptide.

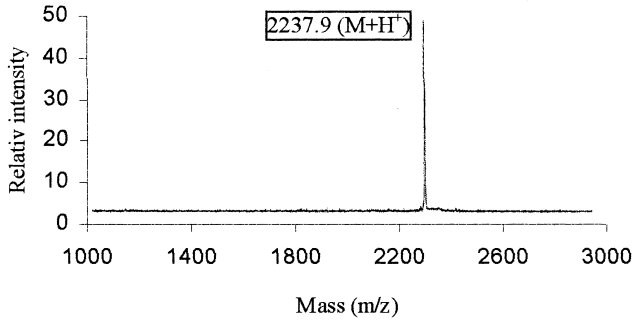

Fig. 2. Mass spectrometry analysis of α 1–23 peptide by MALDI-MS.

The purity of the powder was then checked by analytical RP-HPLC (Fig. 1). The chromatogram shows a single peak corresponding to one pure peptide as assessed by the Millennium software. In order to verify that the peptide obtained was pure and corresponded to the α 1–23 peptide of bovine hemoglobin, mass determination by MALDI-MS and fragmentation by ESI-MS/MS were performed. The mass spectrum is presented in Fig. 2 and shows that the peptide molecular mass is 2236.9 Da. The fragmentation study (not shown) indicated that its amino acid sequence corresponded to the first 23 amino acids in the N-terminus of the bovine hemoglobin α chain: ^{1}VLSAADKGNVKAAWGKVGGHAAE^{23}.

Furthermore, the theoretical p*I* value is 9.86, calculated by the pI/Mw program of ExPASy (http://www.expasy.ch). This value indicated that the peptide is positively charged at pH 7.0.

3.2. Antibacterial activity

The antibacterial activity of the α 1–23 peptide was tested in a liquid growth assay against *M. luteus* strain A270. Growth inhibition of the microbial cells occurred at a concentration of 1.5 mg/ml (671 μM), a concentration corresponding to the MIC. The initial population was 1×10^{6} CFU/ml and after 24 h contact with the peptide, the population was 1×10^{5} CFU/ml in the tube corresponding to the MIC. This result indicates a low bactericidal effect of the peptide at this concentration. Furthermore, when the cell population of the MIC tube was determined, a 72 h incubation of Petri plates was necessary to obtain colonies instead of 24 h for native cells. As many antibacterial peptides act on the permeabilization of the cellular membrane [26], this growth delay on Petri plates of cells previously in contact with the peptide could be due to previous membrane perturbations caused by this peptide.

Recently, the antimicrobial activity of a bovine hemoglobin fragment was described in the gut of the tick *B. microplus* [12]. This fragment, naturally present in the tick, was probably produced by enzymatic cleavage of bovine hemoglobin inside the tick gut. This peptide was active against Gram-positive strains including *M. luteus* A270 and against fungal strains. Its MIC against *M. luteus* A270 was 5 μM, a much lower value than the one obtained with the α 1–23 peptide (670 μM). Nevertheless, the α 33–61 peptide has never been obtained in vitro by enzymatic cleavage.

Although the α 1–23 peptide presents low antibacterial activity compared to other peptides, this is the first report of an antibacterial peptide obtained by in vitro proteolysis of bovine hemoglobin. The mechanism of action of this peptide is probably cellular membrane perturbation leading to growth inhibition of *M. luteus* strain A270.

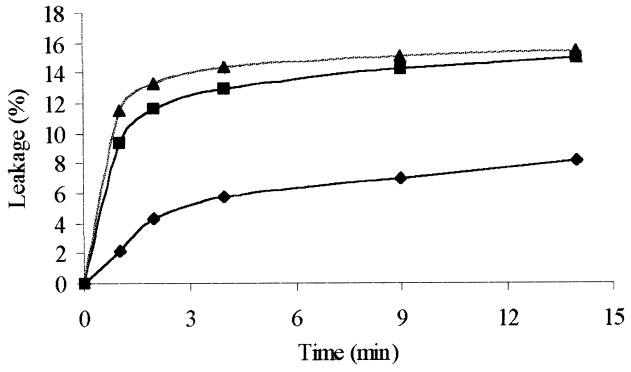

Fig. 3. Time course of carboxyfluorescein leakage from liposomes induced by three concentrations of the α 1–23 peptide. ◆: 25 μM; ■: 37.5 μM; ▲: 50 μM. The PDL induced by the peptides was evaluated using the equation $PDL=[(F-F_0)/(F_t-F_0)]\times100$, where F is the fluorescence intensity after the addition of the peptides, and F_0 and F_t, respectively, the fluorescence intensity without the peptides and after addition of Triton X-100 (2 g/l). Triton X-100 was used to completely release the dye from the liposomes (100% dye leakage).

3.3. Hemolytic assay

The hemolytic activity of the α 1–23 peptide was tested towards bovine erythrocytes. Peptide concentrations of 0.671–3.355 mM were tested. The highest concentration corresponded to five times the MIC against *M. luteus* A270. No hemolysis of erythrocytes was observed with peptide concentrations up to 2.013 mM, a value corresponding to three times the MIC. On the other hand, 11% and 54% hemolysis were observed with peptide concentrations corresponding to four and five times the MIC, respectively. Thus, although the MIC against *M. luteus* A270 was 0.671 mM, this high concentration did not induce lysis of erythrocytes. These results also indicate that the α 1–23 peptide would be non-toxic when used at concentrations lower than 2 mM (three times higher than the MIC), a very high concentration for a peptide.

3.4. Interaction with liposomes and structure of the peptide in solution

To determine if the antibacterial activity of a peptide is due to interaction with cellular membranes, the ability of the peptide to interact with and to disrupt liposomes was studied [26]. Carboxyfluorescein leakage from liposomes induced by the α

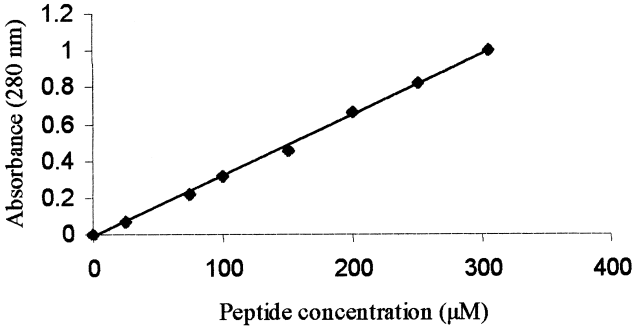

Fig. 4. Relation between absorbance (280 nm) and α 1–23 peptide concentrations in ammonium acetate buffer (10 mM; pH 7.0).

Table 1
Estimation of α 1–23 peptide secondary structure (part of the helix, unordered, and turn structures) in ammonium acetate buffer (20 mM; pH 7.0)

Peptide concentration (w/v, %)	Unordered structure (1620–1650 cm^{-1}, %)	α-Helix (1656 cm^{-1}, %)	Turn (1660–1690 cm^{-1}, %)	Residue (%)
1.25	57	20	19	4
2.5	58	21	20	1
5	62	18	18	4

1–23 peptide at three different concentrations was observed (Fig. 3). The lytic activity of the peptide increased with the concentration to reach 16% leakage at a peptide concentration of 50 μM. This result indicates an interaction between the peptide and liposomes which are structures mimicking bacterial membranes. This interaction with the cellular membranes of the target cells could cause modifications of membrane permeability which can lead to cellular lysis [27,28].

The lytic activity towards the liposomes was weak when compared to natural antibacterial peptides such as melittin which causes at least 80% leakage at a concentration of 100 μM [29]. Nevertheless, such a low result of leakage has already been observed with another biological peptide [30] and this result is in concordance with the high value of the MIC against *M. luteus* strain A270, indicating a low antibacterial activity of the peptide in liquid growth assay.

The relationship between the concentrations of the peptide and the absorbances observed at 280 nm is shown in Fig. 4. The linearity of this relationship lets us suppose that the peptide is present in a unique form. Moreover, exclusion chromatography was performed and the mass obtained for the peptide was approximately 2200 Da (not shown). As the exact mass of the α 1–23 peptide is 2236 Da, the peptide is a unique and monomeric form even at concentrations as high as 10 mg/ml in ammonium acetate buffer (10 mM, pH 7). These results obtained by spectrometry and gel filtration indicated that the α 1–23 peptide presents a low propensity to aggregate in aqueous solution.

The structure in solution of the α 1–23 peptide was then studied by infrared spectroscopy to estimate its secondary structure. The results are presented in Table 1. The α-helical content of the α 1–23 peptide was approximately 20%, which means a low α-helical structuring in comparison to its α-helical content in hemoglobin which is 87% as observed with the Rasmol software. This 20% α-helical content is also slightly weaker but comparable to the average value of 30% α-helical content obtained with different mathematical models from the Network Protein Sequence analysis internet server of the Pôle Bio-informatique Lyonnais (http://pbil.ibcp.fr). Furthermore, these different models indicate a central position of the α-helix for the α 1–23 peptide. As it is partially α-helically structured in ammonium acetate buffer (10 mM, pH 7.0) and also α-helically structured when integrated in bovine hemoglobin, it could be supposed that this peptide would contain a higher α-helical structure than 20 or 30% in an environment mimicking lipid bilayer. Indeed, many antibacterial peptides adopt a random structure in aqueous solution but a mainly α-helical conformation in dodecylphosphocholine or in SDS micelles [31]. This α-helical conformation of antibacterial peptides is essential with regard to their mechanism of action towards the microorganisms. Moreover, many antimicrobial peptides present a high p*I* value. Then, by adopting an α-helical structure, interactions between positively charged peptides and the cellular membranes lead to the formation of channels or pores within the microbial membrane to permeate the cell and impair its ability to carry out metabolic processes [32]. As the α 1–23 fragment is amphiphilic, α-helically structured and positively charged at pH 7.0, this peptide possesses many physicochemical properties of antibacterial peptides.

This peptide, easily purified by a two-step purification process including anion exchange chromatography and preparative RP-HPLC, can be obtained in a relatively high quantity in this way. Furthermore, this soluble peptide could be used at concentrations at least three times the MIC against *M. luteus* strain A270. In the current context of food safety and food protection by means of natural products, use of antibacterial peptides derived from agricultural proteins would be a relevant challenge.

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