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Production of an antimicrobial peptide derived from slaughterhouse by-product and its potential application on meat as preservative

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ABSTRACT

Bovine cruor, a slaughterhouse by-product, contains mainly hemoglobin, broadly described as a rich source of antimicrobial peptides. In the current context of food safety, bioactive peptides could be of interest as preservatives in the distribution of food products. The aim of this work was to study the α137–141 fragment of hemoglobin (Thr-Ser-Lys-Tyr-Arg), a small (653 Da) and hydrophilic antimicrobial peptide. Its production was fast, with more 65% finally produced at 24 h already produced after 30 min of hydrolysis with pepsin. Moreover, increasing substrate concentration (from 1 to 8% (w/v)) resulted in a proportional augmentation of α137–141 production (to 807.95 ± 41.03 mg L^{-1}). The α137–141 application on meat as preservative (0.5%, w/w) reduced the lipid oxidation about 60% to delay meat rancidity. The α137–141 peptide also inhibited the microbial growths under refrigeration during 14 days. These antimicrobial effects were close to those of the butylated hydroxytoluene (BHT).

1. Introduction

Recently, the pharmaceutical and food industries have taken interest in peptides derived from proteins (Bah, Bekhit, Carne, & McConnell, 2013; Castro & Sato, 2015; Clare & Swaisgood, 2000; Danquah & Agyei, 2012; Hartmann & Meisel, 2007). In the current context of food safety, the antimicrobial peptides derived from industrial wastes could be used as preservatives for the storage and distribution of food products (Agyei & Danquah, 2011; Lafarga & Hayes, 2014). The main benefit is to add a value to proteins from wastes (Bah et al., 2013; Goot et al., 2016; Lafarga & Hayes, 2014). Then, antimicrobial peptides represent a promising alternative to synthetic preservatives. Moreover, some of them, like butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT), are suspected to induce pathological and toxic effects (Bauer, Dwyer-Nield, Hankin, Murphy, & Malkinson, 2001; Imaida et al., 1983; Lanigan & Yamarik, 2002). However, these synthetic additives are widely used to protect food, reducing lipid oxidation and microbial growths during food storage. These two last factors are typically involved in the reduction of meat shelf-life and rancidity (Chaâbane et al., 2014; Kim, Cho, & Han, 2013; Weiss, Gibis, Schuh, & Salminen, 2010). Therefore, the peptides derived from food proteins could be natural potential candidates for the substitution of the synthetic compounds (Sakanaka, Tachibana, Ishihara, & Juneja, 2005; Sun, Luo, Shen, Li, & Yao, 2012; Wang, Huang, Chen, Huang, & Zhou, 2015).

In this context, bovine blood is a slaughterhouse waste and its valorization could be interesting by producing antimicrobial peptides (Bah et al., 2013; Lafarga & Hayes, 2014). Raw bovine blood is essentially divided into two by-products after centrifugation: plasma, the colorless part, and cruor, the red fraction. The plasma valorization is effective due to its high protein content, like thrombin, fibrinogen, immunoglobulin G or bovine serum-albumin (Bah et al., 2013). About cruor, it is the fraction which gives blood its red color, representing 45% of the whole blood, and it contains mainly hemoglobin, a globular tetramer (64,500 Da) composed of four protoporphyrin groups, each including an iron atom in their center. This protein is broadly described as a rich source of bioactive peptides after enzymatic hydrolysis (Adje et al., 2011; Gomes et al., 2010; Nedjar-Arroume et al., 2008). The most described activity is antimicrobial (Choisnard et al., 2002; Daoud et al., 2005; Froidevaux et al., 2001). Nevertheless, the peptide production is often poorly mastered or controlled and represents a limiting factor for bioactive peptides generation at large scale or high concentrations.

Our study is focused on the α137–141 fragment (Thr-Ser-Lys-Tyr-Arg), a small (653 Da) and hydrophilic peptide with two

Abbreviations: $A_{\alpha 137-141}$, α137–141 area peak; BH, bovine hemoglobin; BHT, butylated hydroxytoluene; $C_{\alpha 137-141}$, α137–141 concentration; C_{BH}, bovine hemoglobin concentration; CFU, colony-forming unit; DH, degree of hydrolysis; MA, match angle; MT, match threshold; PA, purity angle; PT, purity threshold; RP-HPLC, reversed-phase high performance liquid chromatography; TBARS, thiobarbituric acid-reactive substances.

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positive charges at pH 7 (Catiau et al., 2011), which has a large antibacterial spectrum, especially against pathogenic bacteria commonly responsible for food alteration, such as *Escherichia coli*, *Salmonella enterica* or *Staphylococcus aureus* (Borch & Arinder, 2002; Catiau et al., 2011). However, many studies have shown the enzymatic mechanism involved in the peptide generation for the purified bovine hemoglobin. Hence, the α137–141 production is not investigated into the cruor, the by-product slaughterhouse. Moreover, for its potential valorization, it is important to control the process at high initial substrate concentrations. Additionally, it is necessary to quantify the α137–141 generation during the course of hydrolysis.

Hence, the first aim of this work was to study the α137–141 production during the bovine cruor hydrolysis to understand the involved mechanisms. Its production quantification was investigated for several substrate concentrations (from 1 to 8%, w/v). Therefore, the α137–141 application as food preservative was studied and the α137–141 antimicrobial effects were identified on meat during food storage.

2. Materials and methods

2.1. Reagents, solvents and standards used

All chemicals and solvents were of analytical grade from commercial suppliers: Sigma-Aldrich (Saint-Quentin Fallavier, France) or Flandres Chimie (Villeneuve d'Ascq, France). The ultrapure water was prepared using a Milli-Q system. Bovine cruor was supplied by Vapran SA (Plemet, France). The standard α137–141 was provided by Genecust (Luxembourg). The beef meat was obtained from a local purveyor.

2.2. Hydrolysate preparation

2.2.1. Stock solution preparation

A stock solution was prepared by adding 15 g of purified bovine hemoglobin (BH) or bovine cruor into 100 mL of ultrapure water. After centrifugation at 4000 min^{-1} for 30 min, the supernatant was recovered and the real BH concentration (C_{BH}) was dosed with the Drabkin's method established by Crosby, Munn, and Furth (1954). After that, the stock solution of purified bovine hemoglobin or bovine cruor was diluted to obtain a solution with a given C_{BH}: 1, 2, 5 and 8% (w/v).

2.2.2. Hydrolysis process

In the stock solution, the BH was native. To denature the hemoglobin without the addition of a chemical denaturant, the hydrolysis took place in sodium acetate and acetic acid buffer (0.5 M) at pH 3.5 (Dubois, Nedjar-Arroume, & Guillochon, 2005). The BH was digested by pepsin from porcine gastric mucosa (EC 3.4.23.1, 3200–4500 units mg^{-1} protein) which was prepared in acetic acid and sodium acetate buffer (0.1 M; pH 3.5) at 23 °C, with the ratio enzyme/substrate = 1/11 (mole/mole). Sampling was performed during the hydrolysis at 0, 1, 2.5, 5, 10, 15, 30 min and 1, 2, 3, 10 and 24 h, corresponding to different degrees of hydrolysis. The peptic hydrolysis was stopped by addition of sodium hydroxide 5 M up to a final pH of 10 which denatured the enzyme. The samples were placed at −20 °C to avoid basic hydrolysis. They have been carried out in triplicate.

2.3. Determination of the degrees of hemoglobin hydrolysis

The degree of hydrolysis (DH) is defined as the ratio of the number of peptide bonds cleaved to the total number of peptide bonds of BH (i.e. 568) and is expressed in percentage. The cleavage of peptide bonds was adapted from the *ortho*-phthaldialdehyde (OPA) method by Church, Swaisgood, Porter, and Catignani (1983).

2.4. RP-HPLC analyses

2.4.1. Materials, software and elution program used

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 an NEC image 466 computer. Millenium software was used to plot, acquire and analyze chromatographic data. All the chromatographic processes were performed with a Vydac C_4 column (250 mm × 4.6 mm, internal diameter of 3 mm). The mobile phases were ultrapure water/trifluoroacetic acid (1000:1, v/v) as eluent A, and acetonitrile/ultrapure water/trifluoroacetic acid (600:400:1, v/v/v) as eluent B. Samples were filtered through 0.20 μm filters and then injected. Online UV absorbance scans were performed between 200 and 390 nm at a rate of one spectrum per second with a resolution of 1.2 nm. Chromatographic analyses were completed with Millenium software (Choisnard et al., 2002; Zhao, Piot, Gautier, & Cottenceau, 1996). The injection volume was 10 μL. The flow rate was 0.6 mL min^{-1}. The gradient applied was 100% (v/v) A over 5 min, 0–67% (v/v) B over 30 min, then 67–87% (v/v) B over 35 min.

2.4.2. Spectral comparison and assessment of peak purity by peptide spectral library

Rapid identifications of expected peptides from the very complicated hemoglobin peptic hydrolysates were carried out by UV-spectral comparison as described previously for the haemorphins (Choisnard et al., 2002; Zhao, Sannier, Ricart, & Piot, 1995). The spectrum of the standard peptide was taken as a norm in the Millenium library. Two types of mathematical analyses were established: an assessment of peak purity and a comparison between the spectrum of sample peak and the standard spectrum. Mathematical analyses were quantitatively expressed using the following criteria.

For the differences between any two spectra, two criteria were expressed. (I.i) The Match Angle (MA) is a measurement of the difference in spectral shapes between an unknown spectrum and a library spectrum. The MA can range from 0 to 90 degrees. Lower values indicate that spectra are similar. Larger values indicate greater degrees of spectral difference. (I.ii) The Match Threshold (MT) indicates the sensitivity of the measurement. It can range from 0 to 180 degrees. Larger values indicate lower measurement sensitivity. In general, if the MA is higher than the MT, it indicates that the two spectra are different. If the MA is lower than the MT, it indicates that the two spectra are the same or similar (Zhao et al., 1995). Therefore, identical spectra allow us to identify expected peptides.

Nevertheless, a second measurement was applied. Two criteria were expressed to assess the peak purity from chromatographic profiles. (II.i) The Purity Angle (PA) represents the relative spectral homogeneity across the peak for each purity pass. This value can range from 0 to 90 degrees with 0 indicating a perfect spectral homogeneity. The Purity Threshold (PT) includes noise, high sample concentration, photometric error and/or solvent and may cause a largest PA which is calculated for each purity pass. If the PA is lower than the PT, spectra are homogeneous within the noise of measurements, but that cannot be used to prove chemical purity.

For each measurement, the lower values of each parameter were researched, respecting the MA lower than the MT and the PA lower than the PT. With that, the α137–141 measurement was the most realistic and avoided the inclusion of other compounds in the assessment of α137–141 quantity from samples.