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Antibacterial peptides from barbel muscle protein hydrolysates: Activity against some pathogenic bacteria

Assaâd Sila [a,b,*], Naima Nedjar-Arroume [b], Karima Hedhili [b], Gabrielle Chataigné [b], Rafik Balti [c], Moncef Nasri [d], Pascal Dhulster [b], Ali Bougatef [e]

[a] Unité Enzymes et Bioconversion, Ecole nationale d'Ingénieurs, PB 1173, Sfax 3038, Tunisia
[b] Laboratoire de Procédés Biologiques, Génie Enzymatique et Microbien, IUT A Lille I, BP 179, Villeneuve d'Ascq Cedex 59653, France
[c] Institut Supérieur de Biologie Appliquée, Médenine 4119, Tunisia
[d] Laboratoire de Génie enzymatique et de Microbiologie, Ecole nationale d'Ingénieurs, PB 1173, Sfax 3038, Tunisia
[e] Institut Supérieur de Biotechnologie de Sfax, BP 1175, Sfax 3038, Tunisia

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ABSTRACT

Peptides obtained by enzymatic hydrolysis of fish proteins exhibit not only nutritional but also biological properties of dietary uses, or even therapeutic potential. The objective of the present study was to isolate and characterize peptides from the protein hydrolysates of barbel muscle with antibacterial activity against Gram-positive (*Listeria monocytogenes*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Micrococcus luteus* and *Bacillus cereus*) and Gram-negative (*Escherichia coli*, *Salmonella enterica*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Enterobacter* sp.) bacteria. Barbel muscle protein hydrolysates (BMPHs), obtained by treatment with Alcalase® (DH = 6.6%), was fractionated by size exclusion chromatography on a Sephadex G-25 and purified by reversed-phase high performance liquid chromatography (RP-HPLC). The molecular masses and amino acid sequences of these peptides were determined using ESI–MS and ESI–MS/MS, respectively. Eleven peptides in F_{II-1}, F_{II-2}, F_{II-3} and F_{II-4} sub-fractions separated by RP-HPLC were identified. The most active peptide fraction (F_{II-3}) contained three peptides: Ala–Ala–Ala–Leu; Ala–Ala–Gly–Gly–Val and Ala–Ala–Val–Lys–Met.

These peptides don't show hemolytic activity towards bovine erythrocytes. These results suggest that some peptides from barbel could be a beneficial ingredient for nutraceuticals.

1. Introduction

Antimicrobial peptides (AMPs) are important components of the innate immune systems of countless animal species, where they act as an effective, largely non-discriminatory first line of defense against invading pathogens (Brogden, 2005; Jenssen, Hamill, & Hancock, 2006; Yeaman & Yount, 2003). Spurred by the proliferation of bacterial strains that exhibit resistance to conventional antibiotics, the last two decades have seen a flurry of research activities aimed at developing novel therapeutic antibiotics based on AMPs.

The natural AMPs have been isolated and characterized from practically all-living organisms, ranging from prokaryotes to humans. The AMPs produced by bacteria are also termed 'bacteriocins' (Boulanger, Bulet, & Lowenberger, 2006; Brogden, 2005). AMPs usually work against bacteria that are closely related to the producer strains in prokaryotes, while they play a key role in innate immunity in eukaryotes. These peptides are produced by several species including bacteria, insects, plants, vertebrates and they have been recognized as ancient evolutionary molecules that have been effectively preserved in mammals (Altincicek, Linder, Linder, Preissner, & Vilcinskas, 2007; Konno et al., 2007). The significant advantage of AMPs resides in the global mechanism of their action, which is remarkably different from that of conventional antibiotics. Usage of AMPs will gain widespread increase since more and more bacteria may develop the ability to resist conventional antibiotics due to the abuse of these drugs worldwide. Since the first AMP Cecropin was discovered from *Hyalophora cecropia* in the 1980s by Boman's research group, a large number of antimicrobial peptides, which share common features such as relatively low molecular weight, positive charge and inducibility by injury and microorganism infection, have been identified (Steiner, Hultmark, Engstrom, Bennich, & Boman, 1981).

Since the ninety, several peptides with antibacterial activities against Gram-positive and Gram-negative bacteria were identified

* Corresponding author. Unité Enzymes et Bioconversion, Ecole nationale d'Ingénieurs, PB 1173, Sfax 3038, Tunisia. Tel.: +216 97 013 118; Fax: +216 74 275 595.
E-mail address: assaadsila@gmail.com (A. Sila).

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and isolated in protein hydrolysates. The nature of these proteins is very diverse and includes casein (Zucht, Raida, Adermann, Mägert, & Forssmann, 1995), ovotransferrin (Ibrahim, Iwamori, Sugimoto, & Aoki, 1998), lactoferrin (Bellamy et al., 1992), and β-lactoglobulin (Pellegrini, Dettling, Thomas, & Hunziker, 2001). While the majority of antibacterial peptides, produced by enzymatic hydrolysis of proteins, were isolated from milk proteins (Pellegrini, 2003). Several studies have reported the obtaining of antibacterial peptides by enzymatic hydrolysis of hemoglobin (Yaba Adje et al., 2011). Antibacterial peptides derived from marine organisms by enzymatic treatment have been isolated (Abidi et al., 2013; Beaulieua, Thibodeaua, Bonnet, Bryl, & Carbonneauc, in press; Doyena, Saucier, Beaulieua, Pouliot, & Bazinet, 2012).

The barbel, *Barbus callensis*, is a group of small carp-like fish of the genus *Barbus* that has a wide distribution in northern and central Tunisia.

As far as we know, no studies have analysed the antibacterial activity of barbel protein hydrolysates in vitro and in vivo. In this study, we investigated the antibacterial activity of barbel muscle protein hydrolysates obtained by treatment with Alcalase®. The amino acid sequences of peptides in most active sub-fractions were determined.

2. Materials and methods

2.1. Reagents

Common chemicals and solvents of analytical grade were obtained from different commercial sources. Alcalase® 2.4 L serine-protease from *Bacillus licheniformis* was supplied by Novozymes® (Bagsvaerd, Denmark). Sephadex G-25 was purchased from Pharmacia (Uppsala, Sweden). Water was obtained from a Culligan system; the resistivity was approximately 18 MΩ. All other chemicals and reagents used were of analytical grade.

2.2. Enzyme

The serine protease from *B. licheniformis* (Novozymes®) was used for the production of hydrolysates. Protease activity was determined according to the method of Kembhavi, Kulkarni, and Pant (1993) using casein as a substrate. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine per minute under the experimental conditions used.

2.3. Sample preparation

The barbel (*B. callensis*) samples used in the present work were obtained from Barrage SIDI SAAD, Kairouan, Tunisia. The samples were packed in polyethylene bags, placed in ice (sample/ice ratio of about 1:3 (w/w)), and transported to the laboratory within 2 h after collection. The internal organs were separated and then stored in sealed plastic bags at −20 °C.

2.4. Preparation of barbel muscle protein hydrolysates (BMPHs)

Barbel muscle (500 g), in 500 ml distilled water, was first minced using a grinder (Moulinex Charlotte HV3, France) then cooked at 90 °C for 5 min to inactivate endogenous enzymes The cooked muscle sample was then homogenised in a Moulinex® blender for about 2 min. The sample was adjusted to optimal pH and temperature for Alcalase® (pH 8.0; 50 °C). The hydrolysis reaction was started by the addition of the enzyme at a 1:1 (U/mg) enzyme/protein ratio. The reaction was conducted at 50 °C and pH 8.0 for 2 h. During the reaction, the pH of the mixture was maintained at the desired value by continuous addition of 4 N NaOH. After the required digestion time, the enzymatic hydrolysis was stopped by addition of disodium tetraborate (0.32 M, pH 12.7) up to a final pH of 12. Protein hydrolysates were then centrifuged at 5000 *g* for 20 min to separate soluble and insoluble fractions. Finally, the soluble fractions, referred to as protein hydrolysates, were freeze-dried at −50 °C and 121 mbar (Modulyod-230, Thermo-Fisher Scientific, Waltham, MA) and then stored at −20 °C for further use.

2.5. Determination of the degree of hydrolysis

The degree of hydrolysis (DH), defined as the percent ratio of the number of peptide bonds cleaved (h) to the total number of peptide bonds in the substrate studied (h_{tot}), was calculated from the amount of base (NaOH) added to keep the pH constant during the hydrolysis (Adler-Nissen, 1986) according to the following equation.

$$\mathrm{DH}\% = \frac{h}{h_{tot}} \times 100 = \frac{B \times \mathrm{Nb}}{\mathrm{MP}} \times \frac{1}{\alpha} \times \frac{1}{h_{tot}} \times 100$$

where B is the amount of NaOH consumed (ml) to keep the pH constant during the proteolysis of the substrate. Nb is the normality of the base, MP is the mass (g) of the protein ($N \times 6.25$), and α represents the average degree of dissociation of the α-NH_2 groups in the protein substrate expressed as:

$$\alpha = \frac{10^{\mathrm{pH}-\mathrm{pK}}}{1 + 10^{\mathrm{pH}-\mathrm{pK}}}$$

Where pH and pK are the values at which the proteolysis was conducted. The total number of peptide bonds (h_{tot}) in the protein substrate was assumed to be 8.6 meq/g (Adler-Nissen, 1986).

2.6. Purification of the antibacterial fractions by HPLC

The lyophilized hydrolysate (1 g), with a DH of 6.6%, obtained by treatment with Alcalase® for 15 min, was suspended in 5 ml of distilled water, then separated onto a Sephadex G-25 gel filtration column (5.2 × 56 cm) pre-equilibrated and eluted with distilled water. Fractions (4 ml) were collected at a linear flow rate of 30 ml/h. Fractions (4 ml each) were collected and elution curves were obtained by measuring absorbance at 226 nm using an online spectrophotometer. Fractions that showed antibacterial activity were pooled and lyophilized.

The most active fraction was dissolved in Milli Q water, filtered through 0.22 μm filters, and then separated by Reverse-phase high-pressure liquid chromatography (RP-HPLC) on a Waters C_{18} column (4.6 mm × 250 mm) (XBrideg™, Ireland). Sample was injected at a volume of 60 μl (50 mg/ml). Peptides were eluted with eluent A (water containing 0.1% trifluoroacetic acid (TFA)) for 5 min, then with a linear gradient of acetonitrile (40–100% for 60 min) containing 0.1% TFA at a flow rate of 1 ml/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. The antibacterial activities of the eluted peaks were determined. 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 analyse chromatographic data.

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