Biochimica et Biophysica Acta 1828 (2013) 1066–1074

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Structural and functional studies on a proline-rich peptide isolated from swine saliva endowed with antifungal activity towards *Cryptococcus neoformans*

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ARTICLE INFO

Article history:
Received 5 June 2012
Received in revised form 11 December 2012
Accepted 14 December 2012
Available online 26 December 2012

Keywords:
Proline-rich peptide
Antifungal
Circular dichroism
Membrane integrity
Polyproline-II
Cryptococcus neoformans

ABSTRACT

A proline-rich peptide of 2733 Da, isolated from pig parotid granule preparations was tested against different pathogenic fungi. It showed interesting antifungal activity towards a clinical isolate of *Cryptococcus neoformans*, with an EC_{50} of 2.2 μM. Neither cytotoxic nor haemolytic effects were observed towards mammalian cells. Circular dichroism and infrared spectroscopic studies showed that the peptide adopted a combination of polyproline type-II, β-turn and unordered conformations at physiological temperatures. Temperature dependent experiments evidenced a tendency to adopt a polyproline-II helix conformation. From experiments with lipid vesicles, Neutral Red Uptake (NRU), haemolytic assays, and confocal microscopy studies, it could be hypothesized that the peptide may exert its antifungal effect by interacting with an intracellular target rather than through membrane damage.

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1. Introduction

Cryptococcosis is a serious and potentially life-threatening disease caused by environmental yeasts belonging to the genus *Cryptococcus*. Only *Cryptococcus neoformans* together with *C. gattii* are considered, among the many recognized species in the genus, the principal pathogens in humans. Previously, *C. neoformans* was defined as having two varieties — var. *neoformans* and var. *gattii*. They differed on the basis of antigenic specificity of the capsular polysaccharide: serotypes A, D and AD were recognized in var. *neoformans*, and serotypes B and C in var. *gattii*. Serotype A strains have been afterwards named *C. neoformans* var. *grubii* [1]. However, based on the elucidation of the genomic sequences, *C. gattii* is now considered a distinct species. Infection begins in the lung, following inhalation of environmental yeast cells. Initial manifestations depend likely on the fungal burden and the immune status of the host, and pulmonary cryptococcosis varies from a benign upper respiratory tract infection to a very severe bilateral pneumonia. The infection can spread through hematogenous dissemination. Cryptococci have a predilection to invade the central nervous system and can cause a life-threatening meningoencephalitis with involvement of motor and cognitive functions. Although cryptococcosis is mainly observed in cases of immune deficiency, many reports highlight its occurrence in patients without recognizable immune defects, indicating that the yeast can set up virulence mechanisms that provoke disease even in healthy individuals [2,3]. HIV-infected individuals are particularly prone to cryptococcal infection; cryptococcosis is an AIDS-defining illness and a major cause of mortality, particularly in sub-Saharan Africa [4]. The treatment regimens for cryptococcal meningitis are focused on amphotericin B, alone or in combination with flucytosine, for initial or induction treatment. Azoles, as fluconazole or itraconazole, remain the agents of choice for long-term maintenance therapy, to prevent relapses or as available therapeutic alternative. As for many other infectious diseases, treatment failures may occur because of antifungal drug resistance [5,6], so the development of new drugs with different mechanisms of action, including peptides endowed with antifungal activity [7,8], has to be pursued in order to face more threatening fungal infections in the future.

In this view, over the past decades a great interest has focused on antimicrobial peptides (AMPs) [9,10]. AMPs are a large and heterogeneous

Abbreviations: SPPS, (Solid Phase Peptide Synthesis); MALDI-TOF, (Matrix Assisted Laser Desorption Ionization – Time of flight); CD, (Circular Dichroism); ATR/FT-IR, (Attenuated Total Reflectance/Fourier Transform-Infra Red); PDA, (Polydiacetylene)

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http://dx.doi.org/10.1016/j.bbamem.2012.12.013

family of peptides widespread in plant and animal kingdoms as important effectors of innate immunity. They have several common features, including the presence of a basic character with a positive net charge at physiological pH. AMPs are divided into sub-families depending on secondary structural similarities: the alpha-helical conformation is a common motif in the secondary structure of melittin, magainin, and the cathelicidin LL-37 [11]; beta strands with one or more disulfide bonds are common in defensins, cystatins, hepcidins, and thionins [12–15], while apidaecins, indolicidins, and histatins share a linear structure and are also characterized by the presence of specific amino-acids (e.g. Arg, Pro, His) [16,17]. Penaeidins, isolated from crustaceans, exhibit a peculiar structure being constituted by two distinct domains: a linear proline-rich and a cysteine rich one [18]. AMPs show anti-bacterial, antiviral and antifungal activities at a concentration ranging from nano to micromolar, and some of them are under development as new promising drugs [19]. Of particular interest is the fact that each class may exert killing activity through mechanisms of action different with respect to conventional antimicrobial drugs. Depending on experimental conditions, these mechanisms may be included in two main schemes: a) microbe cell membrane perturbation or disruption; b) interaction with intracellular targets [20].

A peculiar class of AMPs is represented by proline-rich peptides. They are small linear peptides characterized by a high content (up to 50%) of proline residues [21]. The most well known representative members of this class are the mammalian cathelicidins Bac-5, Bac-7 and PR-39 [22–24], crustacean penaeidins and insect apidaecins, but peptides derived from gastropods and earthworms were also described [25,26].

Due to their structural arrangement, the modalities by which proline-rich peptides exert their antimicrobial activity reside in the ability to translocate across the target organism membrane and interact with intracellular targets [27]. These are represented by DNA, enzymes, or protein complexes. In the latter case, peptides can interact with Proline-Rich Sequence Recognition Domains, such as Src Homology 3 (SH3) domains, being able to modulate different cellular mechanisms and pathways [28–30]. Proline-rich peptides are present in different body compartments and fluids in mammals, and saliva is one of the richest sources of these peptides in the mammalian order. Proteomic investigation of the pig saliva revealed the presence of interesting proline-rich peptides among which the main component, named SP-B peptide, showed to possess antifungal activity [31]. The aim of this study was to investigate the biological activities against pathogenic fungi of another minor component identified in swine saliva [32] and recently patented as anti-viral agent [pat. n° PCT/IB2012/050419].

2. Materials and methods

2.1. Peptide synthesis

SP-E peptide was assembled on an Applied Biosystem Peptide Synthesizer 433A (Foster City, CA, USA) on a preloaded proline-2-chlorotrityl resin (Novabiochem, Laufelfingen, CH) following the Fmoc-(N^{α}-9-Fluorenylmethyloxycarbonyl) protocol for stepwise solid phase peptide synthesis [33,34]. Fmoc-amino acids were from Novabiochem.

All couplings were carried out with 5 fold excess of activated amino acid in the presence of 10 equivalents of *N*-ethyldiisopropyl amine, using *N*-[(dimethylamino)-1-*H*-1,2,3-triazole-[4,5-β]pyridine-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU, PE Biosystems, Inc., Warrington, UK) as activating agent for the carboxy group. The fluoresceinated peptide was obtained by extending the N-termini of an aliquot (15%) of the assembled peptide-resin, with 8-(9-Fluorenyloxycarbonyl-amino)-3,6-dioxaoctanoic acid, removal of the Fmoc protecting group and coupling of 5-Carboxyfluorescein (FAM), mediated with 1-Hydroxybenzotriazole and *N*, *N*′-Diisopropylcarbodiimide. The fluoresceinated peptide was released from the resin and purified by the same procedures adopted for the free-peptide. At the end of peptide chain assembly, the peptide was cleaved from the resin by treatment with a mixture of 80% trifluoroacetic acid, 5% water, 5% phenol, 5% thioanisole, 2.5% ethandithiol and 2.5% triisopropylsilane for 3 h at room temperature, with concomitant side chain deprotection. The resin was filtered and the peptide was precipitated in cold *tert*-butylmethyl ether. After centrifugation and washing with *tert*-butylmethyl ether the peptide was suspended in 5% aqueous acetic acid and freeze-dried. Analytical and semipreparative Reversed Phase High Performance Liquid Chromatography (RP-HPLC) was carried out on a Tri Rotar-VI HPLC system equipped with a MD-910 multichannel detector for analytical purposes or with a Uvidec-100-VI variable UV detector for preparative purposes (all from JASCO, Tokyo, Japan). Analytical RP-HPLC was performed on a Jupiter 5 μ C18 300 Å column (150 × 4.6 mm, Phenomenex, Torrance, CA, USA). Semipreparative RP-HPLC was performed on a Jupiter 10 μ C18 300 Å column (250 × 21.2 mm, Phenomenex). Linear gradients of acetonitrile in aqueous 0.1% TFA (v/v) were used to elute bound peptide. MALDI-TOF mass spectrometry analysis was performed on a Autoflex workstation (Bruker Daltonics, Bremen, DE). Observed experimental values for peptide masses were in agreement with theoretical calculated values.

2.2. CD and ATR/FT-IR measurements

CD spectra were obtained on a Jasco J-600 spectrophotometer equipped with a thermostatic temperature controller. CD spectra were recorded in quartz cell of 0.1 cm path length at 25 °C between 190 and 250 nm, using a 2.0 nm bandwidth and a scanning rate of 20 nm/min with a wavelength step of 0.1 nm and a time constant of 0.1 s. SP-E peptide was dissolved in 10 mM sodium phosphate buffer at pH 7.4. A TFE 30% (v/v) aqueous solution was also employed. CD band intensities are expressed as molar ellipticities, ($[\theta]_M$ in deg cm^2 $dmol^{-1} \times 10^{-3}$).

ATR/FT-IR spectra were recorded on a Spectrum One (Perkin–Elmer) spectrophotometer equipped with an ATR accessory with a ZnSe reflection element. Prior to analysis, SP-E synthetic peptide was dissolved in 20 mM HCl and subsequently freeze-dried twice in order to remove the residual TFA derived from peptide purification. Spectra were recorded after 40 scans at a 1 cm^{-1} of resolution. The samples were dissolved in 10 mM sodium phosphate buffer, pH 7.4. An open beam background spectrum of clean crystal was recorded. Subsequently the buffer solution spectrum was recorded followed by the peptide spectrum measurement. The spectra of buffers alone were hence subtracted from the peptide ones. During measurements performed at 25 °C, the crystal was continually flushed with nitrogen to eliminate residual water vapors. Usually 1 μL of a 1 mg/mL (w/v) solution of SP-E peptide was employed for any measurement.

2.3. Spectroscopic data treatment

ATR FT/IR spectra were used to obtain second derivative spectra with Peak Fit 4.12 software (Sea Solve Software, Inc., San Jose, CA, USA). A 20% smoothing process, employing the Savitzky-Golay algorithm, was performed and the resulting peaks were used as a reference for the subsequent peak fitting analysis performed with the same program. A linear baseline was employed and Gaussian peaks were produced after an iterative adjustment of data until the SSE statistical parameter was under 1×10^{-4}, indicating a good fitting analysis. The resulting peak areas of Amide I were used to determine the contribution of each secondary structure motif.

Circular dichroism spectra were analyzed employing Selcon3 program available on Dichroweb web site (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml) [35]. The data were inserted as requested by the web site manager in the range between 190 and 250 nm. The obtained results satisfied the three basic selection rules: sum of

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