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Trifluoroacetylated tyrosine-rich D-tetrapeptides have potent antioxidant activity

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ABSTRACT

The term "oxidative stress" indicates a set of chemical reactions unleashed by a disparate number of events inducing DNA damage, lipid peroxidation, protein modification and other effects, which are responsible of altering the physiological status of cells or tissues. Excessive Reactive Oxygen Species (ROS) levels may accelerate ageing of tissues or induce damage of biomolecules thus promoting cell death or proliferation in dependence of cell status and of targeted molecules. In this context, new antioxidants preventing such effects may have a relevant role as modulators of cell homeostasis and as therapeutic agents. Following an approach of peptide libraries synthesis and screening by an $ORAC_{FL}$ assay, we have isolated potent anti-oxidant compounds with well-defined structures. Most effective peptides are N-terminally trifluoroacetylated (CF_3) and have the sequence tyr-tyr-his-pro or tyr-tyr-pro-his. Slight changes in the sequence or removal of the CF_3 group strongly reduced antioxidant ability, suggesting an active role of both the fluorine atoms and of peptide structure. We have determined the NMR solution structures of the active peptides and found a common structural motif that could underpin the radical scavenging activity. The peptides protect keratinocytes from exogenous oxidation, thereby from potential external damaging cues, suggesting their use as skin ageing protectant and as cell surviving agents.

1. Introduction

Under physiological conditions, the human body produces highly reactive molecules, referred to as Reactive Oxygen Species (ROS), which, at low levels, provide beneficial effects by supporting cell proliferation and survival pathways. ROS levels are usually controlled by endogenous antioxidants enzymes such as Super-Oxide Dismutase (SOD), catalase, Glutathione PeroXidase (GPX), and low-molecular-weight scavengers, like glutathione (GSH), uric acid, Coenzyme Q 10 (CoQ10) and Alpha Lipoic Acid (ALA). Even though most of ROS molecules are produced into mitochondria during physiological metabolism, it is well established that they may also generate as cellular response to cytokines as well as to external stimuli including xenobiotics and bacterial attack [1,2]. The "oxidative stress" is a pathological condition rising from an excessive amount of ROS molecules and/or a reduced response of endogenous antioxidant defences. Hyperproduction of endogenous and exogenous ROS can damage nucleic acids, proteins and lipids, and consequently alter their physiological functions. Previous investigations on the effects deriving from altered redox control on cellular pathways have allowed a correlation between ROS-induced damages with several pathological conditions associated with cancer [3,4], genetic diseases [5], atherosclerosis, hypertension, ischemia/reperfusion injury, diabetes mellitus [6],

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neurodegenerative diseases [7–10], rheumatoid arthritis, and ageing, accelerating the senile process of the organism [11]. In fact, ROS can directly interact with crucial signalling molecules that promote the activation of cellular pathways, including MAP kinases, apoptosis signal-regulated kinase 1 (ASK1), PI3 kinase (PTEN), protein tyrosine phosphatases (PTP). ROS also interfere with mediators of ROS homeostasis and antioxidant gene regulators such as thioredoxin (TRX), APE-1/Ref-1, and Nrf-2, mitochondrial oxidative stress, apoptosis, aging (p66Shc), with mediators of iron homeostasis through iron–sulfur cluster proteins (IRE–IRP), and ATM (Ataxia–telangiectasia mutated)- regulated DNA damage response [2]. Since ROS levels are important and finely tune cellular mechanisms under both physiological and pathological conditions, a modulated delivery of anti-oxidant agents may represent a relevant therapeutic approach for protecting biomolecules from structural damages and tissues from oxidizing injuries. Past studies have indeed suggested that antioxidants exogenously supplemented may provide protection from the several oxidative reactions occurring in humans and act as anticancer, anti-inflammatory and antiradical agents [12,13]. Nevertheless, the role played by supplemented antioxidants in eliciting curative effects is still largely controversial [14]. In recent years, an ever-increasing interest has been addressed to peptides from food or marine sources identifying bioactive compounds with antioxidative properties [15–17]. Antioxidants such as α-tocopherol, ascorbic acid or Vitamin E are broadly used as food preservatives to extend shelf-life and to improve quality. Their main role in food is associated with the capacity to scavenge radicals, potentially generated by light or oxygen, which can otherwise damage food components strongly affecting stability and safety [18]. Antioxidants of different origin, including synthetic peptides, are largely employed also in the cosmetic industry as active ingredients to protect skin from oxidative damage and early ageing [19]. N-terminally palmitoylated penta- and tetrapeptides, known as Matrixyl, are among the most known cosmetic products having different biological activities. With the aim of identifying new small peptides with anti-oxidant potential, we have used a combinatorial chemistry approach to prepare and screen a library of 145172 N-terminally modified synthetic D-tetrapeptides by a fluorescence-based Oxygen Radical Absorbance Capacity assay ($ORAC_{FL}$, or simply ORAC). The selected peptides have been structurally characterized to correlate the structure with function and have been tested to assess their ability to prevent oxidation reactions on keratinocytes and their potential use as antioxidant agents against skin ageing.

2. Material and methods

2.1. Peptide library design, synthesis and characterization

Peptide libraries were designed in a simplified format as reported in [20,21]. By this approach, a small set of amino acids is chosen to represent the chemical space occupied by very short peptides which can be seen as precursors or templates of small molecular scaffolds. Preferentially, only non-natural or D-amino acids are included in these sets in order to potentially select enzyme-resistant new compounds or to introduce structural determinants inducing preferred conformations. For this assay, we chose a tetrapeptide library made of 12 amino acids to ensure a sufficiently broad chemical diversity, further increased by the N-terminal carboxylic acids, and to maintain as short as possible the time needed for the iterative screening. The set used here, is reported in **Table S1A**. A detailed description of building blocks, N-terminal groups and of synthetic procedures for the library preparation are reported in the Supplementary Material (see **Table S1B)**. See also [20,21]. Attempts to induce peptide oxidation, for example on the histidine or tyrosine side chains or to induce cross-linking [22], were performed by dissolving peptides at 0.1 mM in 5% and 10% H_2O_2 in water and checking changes of molecular weight by mass spectrometry. Assays were performed on peptides CF_3—CO—y–y–h–p—NH_2 and peptide CF_3—CO–y–y–p–h—NH_2 named **Pox, and Pox3**, respectively (see Table 1). Further attempts were performed on peptides dissolved in 50:50 acetone/water at 0.5 μM and treated as described in the next section for the screening assays. Mass spectra were acquired at different time points by MALDI-TOF mass spectrometry. Spectra after 5, 30, 90 and 150 min were collected in the Reflectron positive mode using a Waters MALDI-TOF mass spectrometer Micro MX operated in the positive Reflectron mode between m/z 400–1000 [23].

2.2. ORAC assay and screening of the peptide library

An ORAC (Oxygen Radical Absorbance Capacity) assay was used to iteratively screen the library. The assay measures the antioxidant potential of a given sample, expressed as its ability to scavenge peroxide radicals generated by the decomposition of the azo-compound 2,2′-azobis(2-amidinopropane)-dihydrochloride (AAPH). Details of the method are reported in the Supplementary Material. The assay was performed according to [24–26] using the libraries at the specified concentrations. Trolox (6-hydroxy-2,5,7,8-tetrametmethylchroman-2-carboxylic acid), a water soluble vitamin E analogue, was used as calibration standard. ORAC activity of the samples was calculated as trolox equivalents [27].

2.3. Circular Dichroism (CD) analysis

CD spectra were recorded on a Jasco J-715 spectropolarimeter on peptide solutions at 2.0×10^{-4} M concentration in 10 mM phosphate pH 7.0 or in acetate buffer, pH 5.0, at 25 °C to evaluate peptide conformations under pH conditions more suitable for NMR analysis. Hellma quartz cells of 1 cm path length were used in the far UV region (190–250 nm). Temperature was regulated by a PTC-348 WI thermostat. Spectra were signal-averaged over three scans and baseline corrected by subtracting a buffer spectrum.

2.4. NMR analysis

NMR characterization of peptides was performed in water at 25 °C. Samples were prepared by dissolving weighted amounts of each peptide in water (spectroscopic purity) adding D_2O (ARMAR, isotopic purity 99.8%) for a final 90/10 ratio v/v. Final concentrations were about 2.5 mM. The measured pH of the resulting solutions was around 4.0. Chemical shifts were referred to internal sodium 3-(trimethylsilyl) propionate 2,2,3,3-d4 (TSP). Details concerning NMR analyses are reported in the Supporting Information. Further experiments by NMR were performed on Pox and Pox3 in organic solvents to highlight the potential occurrence of H-bonds between the peptide's side chains, which could favour the transfer of hydrogen ions or electrons.

2.5. Molecular Dynamics (MD) simulations

All simulations were performed by the GROMACS package [28], version 4.5.5. Details of MD simulations are reported in the Supporting Information.

2.6. Membrane ROS measure

1.6×10^4 HaCaT keratinocytes, maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS under 5% CO_2 at 37 °C, were seeded in 96-well plates and grown for 20 h. The cells were