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Optimisation of the antifungal potency of the amidated peptide H-Orn-Orn-Trp-Trp-NH2 against food contaminants

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ABSTRACT

The design of novel efficient antimicrobial peptides (AMPs) faces several issues, such as cost of synthesis, proteolytic stability or cytotoxicity. The identification of key determinants involved in the activity of AMPs, such as cationicity and amphipathicity, allowed the synthesis of short peptides with optimized properties. An ultrashort peptide made of the sequence H-Orn-Orn-Trp-Trp-NH2 (O3TR) showed antifungal activity against several contaminants from food products. This peptide inhibited the growth of the filamentous fungi *Fusarium culmorum*, *Penicillium expansum* and *Aspergillus niger* within a range of concentration of 12.5–50 μg/ml. In addition, O3TR inhibited the growth of the yeast *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Zygosaccharomyces rouxii*, *Debaryomyces hansenii* and *Kluyveromyces lactis* within the range 12.5–50 μg/ml. A derivative peptide, called C12O3TR, made by the addition of lauric acid at the N-terminus of O3TR was 2- to 8-fold more active than O3TR against every species. In addition to the inhibition of conidial germination, O3TR and C12O3TR killed *F. culmorum* hyphae at 100 and 50 μg/ml respectively.

The MIC of the two peptides against *F. culmorum* and *Z. bailii* after heat treatment at 100°C for 60 min and within the pH range 3–10, were not changed. However, the activity of O3TR against *F.culmorum* and *Z. bailii* was strongly reduced in salt solutions, whereas the lauric acid peptide kept its antifungal activity and resistance to proteolytic digestion.

The conjugation with lauric acid reduced the random coiled structure and increased the α-helical content of O3TR. After conjugation with the dye tetramethylrhodamine (TMR), both peptides entered *F. culmorum* spores. They also both induced permeabilization of *F. culmorum* hyphae but only C12O3TR permeabilized *Z. bailii* membrane.

In contrast to the lipopeptide, O3TR did not show haemolytic or cytotoxic activity when applied at the concentrations that exhibited antifungal potency. The two peptides were challenged against a yeast cocktail of *S. cerevisiae* and *Z. bailii*, and *A. niger* in different commercial beverages. After 7 days, O3TR was able to inhibit the yeast cocktail in a commercial lager and carbonated drink. Due to its antifungal potency, high stability and low cytotoxicity, the tetrapeptide could represent a promising starting point of a novel food preservative.

1. Introduction

In order to provide sustainable and safe food, protection from microbiological contamination is essential. Food supply is facing two major issues, due to microbial safety: the risk to human health and economic losses due to food loss. In 2015, foodborne diseases have been estimated by the WHO to affect 1 in 10 people every year and 420,000 cases resulted in death (WHO [World Health Organization], 2015). Beverage industry is not spared by microbial spoilage. Although the low pH of soft drinks prevents most of microbial spoilage, fungal growth still occurrs. Yeasts are the primary contaminants in carbonated products, due to their ability to grow in highly carbonated and with low pH beverages (Tribst et al., 2009; Tserennadmid et al., 2011). In case of poor hygiene, mould can grow thanks to high water availability and acidity (Kregiel, 2015). In addition to health risk, fungal spoilage induces undesirable modifications of flavours and tastes. Chemical preservatives are commonly used to inhibit food spoilage but the intensive overuse of these preservatives has led to a rise of resistant microorganisms. The common preservatives used in soft drinks are weak acid preservatives such as sorbic or benzoic acids (Battey et al., 2002). However, yeast species including *S. cerevisiae* and mostly *Z. bailii* have shown resistance to these acids (Tribst et al., 2009). In addition, the increasing interest of the consumer for products of natural origin generated an increasing development of novel natural antimicrobials.

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Among these natural compounds, antimicrobial peptides (AMPs) are short (12–50 amino acid residues) peptides of the innate immune system found in every kingdom. They contain 2 to 9 basic residues and approximately 50% hydrophobic residues and a diversity of structures. The main advantage of these peptides is their mode of action which, for most of them, does not involve specific targets on bacteria or fungi thereby avoiding the development of resistance (Bechinger and Lohner, 2006; Huang et al., 2010). *In vivo*, apart from their direct action, some cationic antimicrobial peptides (CAPs) are involved in immunomodulatory function reinforcing their antimicrobial potential (Hancock et al., 2016). Several AMPs and AMP-derived lipopeptides derived from AMPs have already been approved by the FDA. However, only the bacteriocin nisin is an antibacterial used as food preservative (Domalaon et al., 2014). Nisin present some limitations, such as its limited spectrum of activity, in the absence of additional treatments (Rydlo et al., 2006).

Positive net charge, hydrophobicity and amphipathicity are the key parameters of the antimicrobial activity of these peptides and many potent peptides have been synthesized based on these properties. However, the high cost of these peptides is a hurdle which led to research on shorter peptides. The shortest peptides are called ultrashort peptides (USPs) whose length is below seven amino acid residues. Among the different USPs, the 4 amino acid H-Orn-Orn-Trp-Trp-NH2 has been characterized (Bisht et al., 2007; Laverty et al., 2010). This peptide displayed antimicrobial activity against several human pathogens (Laverty et al., 2015). The use of conjugated fatty acids has been revealed by different groups to increase the antimicrobial potential and act synergistically with USPs (Laverty et al., 2010; Yadav et al., 2013).

In this study, the *in vitro* activity of the USP H-Orn-Orn-Trp-Trp-NH2 (O3TR) was characterized against some representatives of fungal contaminants found in cereal-based products, such as beer, or soft drinks. In addition to potency and safety, promising antimicrobials in food must present an interesting “cost in use”. Thus, the efficiency of this low-cost 4-residue peptide and a derived lipopeptide to reduce the contamination of pre-inoculated drinks was also determined.

2. Material and methods

2.1. Peptides

The ultrashort amidated tetrapeptide H-Orn-Orn-Trp-Trp-NH2 (O3TR) and its derivatives, (C2H3O)- Orn-Orn-Trp-Trp-NH2 (Ac-O3TR) and a lauric acid ester of O3TR, the lipopeptide (C12H23O)-Orn-Orn-Trp-Trp-NH2(C12O3TR), were synthesized by and purchased from GLBiochem, Shanghai, China. Synthesis was performed by solid-phase peptide synthesis technique on Fmoc MBHA Rink amide resin, followed by removal of the Fmoc group. The purity of all peptides exceeded 90% according to the manufacturer. The lyophilized peptide was reconstituted in deionized water and stored in vials of different concentrations at − 20 °C.

2.2. Fungal strains and growth conditions

The following fungal strains were used in this study: *Fusarium culmorum* FST 4.05, *Aspergillus niger* FST 4.21, *Penicillium expansum* FST 4.22. These fungi were chosen because they represent some of the main contaminants of beverages (Kregiel, 2015). The three strains originated from the culture collection of School of Food and Nutritional Sciences, University College Cork (Cork, Ireland). Spore suspensions were prepared according to Oliveira et al. (2012). Briefly, fungal mycelium was cultured on Potato Dextrose Agar (PDA, Sigma-Aldrich) plates at 28 °C for 7 days. Following incubation, hyphae was inoculated in nutrient-poor broth until sporulation. Fungal spores were collected by filtration (30 μm), counted on a haematocytometer and inoculated in fresh nutrient-poor broth.

The yeast strains were purchased from the Leibniz Institute DSMZ (Braunschweig, Germany) and Puratos (Belgium). *Zygosacharromyces bailii* DSM 70492, *Zygosaccharomyces rouxii* DSM 2531, *Kluyveromyces lactis* DSM 4394 and *Debaryomyces hansenii* DSM 3428, brewing *Saccharomyces cerevisiae* (Fermentis) were chosen since they represent some of the main contaminants of food and beverages products (Wareing and Davenport, 2004). The yeasts were cultured on Yeast Potato Dextrose Agar (YEPD, Sigma-Aldrich) at 25 °C for 2–5 days, depending on the strain.

2.3. Antifungal activity assays

Antifungal *in vitro* assays were conducted as described by Munoz et al. (Muñoz et al., 2014) with some modifications. Briefly, fungal conidia (1×10^4 conidia/ml) were inoculated in synthetic fungi media (SFM) without calcium (50 μM MgSO4/7H2O, 5 μM FeSO4/7H2O, 0.1 μM CoCl2, 0.1 μM CuSO4/5H2O, 2 μM Na2MoO4/2H2O, 0.5 μM H3BO3, 0.1 μM KI, 0.5 μM ZnSO4/7H2O, 0.1 μM MnSO4/1H2O, glucose, asparagine, 20 mg of methionine, 2 mg of myo-inositol, 0.2 mg of biotin, 1 mg of thiamine-HCl, 0.2 mg of pyridoxine-HCl, 0.5 mM K2HPO4/3H2O), as described previously (Thevissen et al., 1999).

Fungal suspension (90 μl) was added to 96-well microplate wells followed by the addition of 10 μl of peptides (0–200 μg/ml). Deionized water was used as negative control. Fungal growth was followed at 595 nm over 96 h at 28 °C on a microplate reader (Multiskan TM, Thermo Scientific). The MIC was defined as the lowest concentration of peptide that showed no growth at the end of the experiment (4 days of incubation) in all the experiments. The IC50 was defined as the concentration required inhibiting 50% of fungal growth and calculated *via* PRISM software based on the microplate data.Each peptide concentration was tested in 3 different wells.

A similar procedure was used for yeasts cells with slight differences. The initial yeast concentration was 1×10^5 cfu/ml in yeast minimal media (YMM), as described previously (Thevissen et al., 1999) and the yeast growth was followed at 25 °C. YMM was made of 0.8 g of complete supplement mixture – URA [CSM-URA] per liter, 6.5 g of yeast nitrogen base (YNB) without amino acids per liter (Formedium, Norfolk, UK) and 10 g of glucose (Sigma-Aldrich) per liter.

2.4. Fungal cell viability assays

Cell viability assays were performed as described by Van Der Weerden et al. (2010) with some modifications. *F. culmorum* hyphae were grown in SFM from a concentration of 5×10^4 spores/ml for 18 h at 28 °C with some modifications. Hyphae were collected by centrifugation (10,000 g for 5 min) and resuspended in fresh medium in an equal volume. Hyphal suspension (90 μl) was transferred in wells of a 96-well microtiter plate with 10 μl of peptides (0–200 μg/ml). Deionized water was used as negative control as described above. After 2 h of incubation at 28 °C, 10 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/ml; Sigma) was added to the wells. After 16 h of incubation at room temperature, medium was removed and 100 μl of MTT solvent (0.1 N HCl in anhydrous isopropyl alcohol) were added to the wells and left for 15 min until cell lysis. The presence of MTT/formazan was monitored by measuring absorbance at 570 nm and subtracting background at 690 nm.

2.5. Modification of fungal cell-wall

In order to identify a potential receptor for the peptides on the fungal cell-wall, the latter was modified as described by Van Der Weerden et al. (2010). *F. culmorum* hyphae were grown from a starting solution of 5×10^4 spores/ml for 18 h at 28 °C. The hyphae were collected by centrifugation (10,000 g for 10 min) and treated with β-glucanase (1 ml of 2 mg/ml) for 1 h at 30 °C. After 3 washings (10 min) in SFM, hyphae were treated with the two peptides as described above. Cell viability assay was monitored using MTT assay after 2 h of