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SHORT COMMUNICATION

Identification and characterization of a novel gene-encoded antioxidant peptide obtained from amphibian skin secretions

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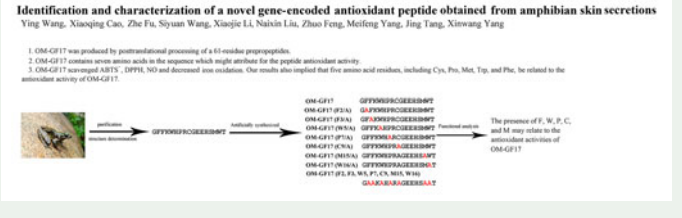
ABSTRACT

Amphibian skin is known to secrete gene-encoded antioxidant peptides of small molecular weight, which play important roles in host defense. However, recognition of such peptides is still in its infancy. Here, we discovered a novel gene-encoded antioxidant peptide (named OM-GF17) from skin secretions of amphibian species, *Odorrana margaretae*. Produced by the post-translational processing of a 61-residue prepropeptide, the amino acid sequence of OM-GF17 was 'GFFKWHPRCGEEHSMWT', with a molecular mass of 2135.7 Da. Functional analysis revealed that OM-GF17 scavenged $ABTS^{+}$, DPPH, NO and decreased iron oxidation. Our results also implied that five amino acid residues, including Cys, Pro, Met, Trp, and Phe, be related to the antioxidant activity of OM-GF17. Furthermore, OM-GF17 did not exhibit direct microbe-killing activity. This novel gene-encoded antioxidant peptide could help in the development of new antioxidant agents and increase our understanding of the biological functions of amphibian skin.



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1. Introduction

Free radicals play crucial roles in the pathogenesis of human diseases, such as inflammation, skin aging, cancer, atherosclerosis, hyperpigmentation, and respiratory diseases (Baur et al. 2006; Calabrese and Maines 2006). Skin serves as a protective barrier and is a major target of oxidative stress (Namjoshi et al. 2008; Yang H et al. 2009). Physical burns and wounds, as well as toxins, UV irradiation, and pathogens, which can directly or indirectly influence metabolic activities, can inflict oxidative stress upon skin (Evelson et al. 1997; Gürbüz et al. 1997; Kohen 1999; Lalhminghlui and Jagetia 2018). Thus, skin is continuously exposed to both endogenous and exogenous sources of free radicals, which need to be controlled and stabilized in the body (Yang H et al. 2009).

Skin has evolutionarily adapted mechanisms to cope with increased oxidative stress, with three antioxidant systems currently recognized (Yang H et al. 2009; Yang X et al. 2016). The first is represented by gene-encoded enzymes with high molecular weight (e.g., ascorbate peroxidase, superoxide dismutase, catalase, and glutathione reductase) (Yang X et al. 2016). The second is composed of non-gene-encoded low molecular weight antioxidants (LMWAs) secreted from the epidermal layer and divided into antioxidants derived from the diet (e.g., vitamin C) and those formed endogenously (e.g., reduced glutathione and ubiquinol-10) (Kohen 1999; Kohen and Gati 2000; Yang X et al. 2016). The third consists of gene-encoded small molecular peptides, which were first identified from amphibian skin except metallothionein (Yang H et al. 2009; Yang X et al. 2016). Until now, a great number of antioxidants, the majority of which belong to the first two antioxidant system, have been identified from many species. However, the information about gene-encoded antioxidant peptide knowledge remains largely unknown, with only a few peptides identified, including antioxidin-RP1 from *Rana pleuraden* (Yang H et al. 2009), antioxidin-I from tropical frogs (Barbosa et al. 2018) and fragilin-A1 and fragilin-B1 from *Limnonectes fragilis* (Yu et al. 2015). Antioxidants, which could scavenge the excess production of free radicals in the body, play important roles in biological system and subsequently prevent free radical-induced diseases (Lalhminghlui and Jagetia 2018). Considering that the identified antioxidants are far from meeting human needs (Gülçin et al. 2010). Thus searching for different types and sources of antioxidants and elucidating their mechanisms of action are still subjects of great interest (Samman 2010).

Amphibian species bridge the evolutionary gap between reptilian and aquatic animals and are broadly distributed worldwide, including high altitude habitats characterized by low temperature and oxygen but high UV radiation (Yang X et al. 2016; Barbosa et al. 2018; Cao et al. 2018). Amphibian skins have therefore acquired a unique molecular mechanism to minimize these environmental threats (Barbosa et al. 2018), and exploration of this mechanism will accelerate our understanding of the novel molecular basis for their defense system.

Odorous frogs are a unique and abundant source of antimicrobial peptides (AMPs) in nature, though many bioactive peptides remain to be identified (Yang X et al. 2012; Cao et al. 2018). In the current study, we identified a novel antioxidant peptide (OM-GF17) from the skin of amphibian species *O. margaretae*. This peptide exhibited $ABTS^{+}$, DPPH, and NO scavenging activity, as well as ferric ions (Fe^{3+}) reducing antioxidant capacity. Meanwhile, we also illustrated that F, W, P, C and M were related to the antioxidant activity. What's more, OM-GF17 did not exhibit direct microbe-killing

activity. Here, we identified a novel gene-encoded antioxidant peptide from amphibian skin, which may help in the development of new antioxidant agents and improve our understanding of the biological functions of amphibian skins.

2. Results and discussion

In this research, we aimed to discover novel antioxidant peptides from amphibian species, *O. margaretae*. Through gel filtration and C18 RP-HPLC purification, a peak that showed $ABTS^+$ scavenging activity (eluted at about 40.6 min as previously reported by Li et al. 2018). This peak was further purified by HPLC, and a final peak with an ideal shape and identical elution time (arrow in Fig. S1) was obtained. Then using Edman sequencing and cDNA cloning we identified a novel peptide (OM-GF17) with a complete amino acid sequence of 'GFFKWHPRCGEEHSMWT' (Fig. S2A). The observed molecular mass (2135.7 Da), as revealed by mass spectrometry, and the theoretical molecular mass (2135.40 Da), as calculated at http://web.expasy.org/compute_pi/, were well-matched (Fig. S2B), indicating there were no post-translational modifications. By Blast searching the NCBI database, this peptide showed 83% sequence similarity to the nigrocin-2S precursor from *O. schmackeri* (Fig. S2C), but contained no intramolecular disulfide bridge. This indicated that OM-GF17 was a novel gene-encoded antioxidant peptide belonging to a novel family. Considering its sequence similarity with antimicrobial nigrocin and the antimicrobial activity of other gene-encoded amphibian peptides (e.g., antioxidin-I) (Barbosa et al. 2018), we speculated that the new peptide might also exhibit antimicrobial activity. However, our results showed that OM-GF17 had did not possess antimicrobial activity (Table S1).

Of note, OM-GF17 demonstrated obvious $ABTS^+$, DPPH, and NO scavenging activity, as well as Fe^{3+} reducing antioxidant capacity, although this activity was slightly weaker compared with other identified odorous frog peptides, such as adersonin-AOP1 (Yang X et al. 2016). As shown in Fig. S3A and C, OM-GF17 showed $ABTS^+$ scavenging activity in both dose- and time-dependent manners. Based on previous study, five amino acids (C, P, M, W, and F) significantly influence the antioxidant activity of peptides (Yang H et al. 2009). Thus, we transformed the structure of the new peptide to elucidate the amino acids that might influence the antioxidant activity of OM-GF17. As shown in Fig. S3B, there was no obvious influence on $ABTS^+$ scavenging activity when the five amino acids were mutated singly; however, the antioxidant activity was eliminated when the five amino acids were mutated together. These results indicated that C, P, M, W, and F played a synergistic role in the peptide antioxidant activity. Previous research has suggested that free C is responsible for the $ABTS^+$ scavenging efficiency of antioxidant peptides (Yang X et al. 2016). We also investigated whether the replacement of the residues had an influence on $ABTS^+$ scavenging efficiency. Compared with OM-GF17, which reached a maximum scavenging rate within 10 s (Fig. S3C), when M15 and W16 were replaced the scavenging rate decreased to ~50 s and when W5 and C9 were replaced the scavenging rate decreased to ~60 s (Fig. S3C). Moreover, the efficiency of the remaining three mutants was much lower, requiring approximately 120 s to reach their maximum scavenging rates (Fig. S3C). These results indicated that apart from the free C, P, M, W, and F also

influenced the $ABTS^+$ scavenging efficiency, and F2, F3, and P7 showed greater responsibility for the scavenging efficiency of OM-GF17. At concentration of 5 μM, OM-GF17 showed slight DPPH scavenging activity, with F2, F3, M15, and W16 responsible for the peptide's DPPH scavenging ability (Fig. S4A). However, when the concentration was decreased to 2.5 μM, OM-GF17 and the mutants DPPH scavenging activity were eliminated (Fig. S4B). For the C9 mutant, the Fe^{3+} reducing power of OM-GF17 was eliminated, whereas the OM-GF17 (F2/A), (F3/A), (W5/A), (P7/A), (M15/A), and (W16/A) mutants showed similar activity as natural OM-GF17 (Table S2). These results demonstrated that C9 was likely responsible for the peptide's Fe^{3+} reducing power. When the F2, F3, or M15 were mutated, the peptide lost its NO scavenging activity. Interestingly, for the five mutated amino acids, OM-GF17 still exhibited NO scavenging activity, indicating that NO scavenging activity may have a different mechanism in compare with $ABTS^+$ and DPPH free radical scavenging (Fig. S5). Considering the above results, we predicted that the antioxidant activity of amphibian peptides might be related to their amino acid composition and sequence, and the amino acids in the sequence may play different roles in $ABTS^+$, DPPH and NO scavenging activities, as well as the Fe^{3+} reducing power. What's more, although C, P, M, W, and F residues are considered to be responsible for antioxidant activity in general, it doesn't necessarily mean that they are all directly participating in the quenching of free radicals. Some of these residues could instead be important for maintaining the active tertiary structure of the peptide. Insights into the structural features originated from circular dichroism spectroscopy, silico modeling and NMR, could help us clarify which residues are directly involved in radical quenching and which are structurally important, however, all these experiments are still awaiting of future study.

In summary, OM-GF17 from *O. margaretae* skin demonstrated the third antioxidant system that is characterized by 'gene-encoded peptides secreted from cells'. Specifically, OM-GF17 was secreted and of gene-encoded origin, the same as antioxidant enzymes, but had no enzyme activity, and it acted as a direct free radical scavenger but with higher molecular weight. Thus, the discovery of OM-GF17 expands our knowledge on the composition of skin secretions from odorous frogs.

3. Conclusions

In this research, a novel gene-encoded peptide was identified from skin secretions of the odorous frog *O. margaretae*. The peptide was named OM-GF17 and presented an amino acid sequence of 'GFFKWHPRCGEEHSMWT'. Functional analysis revealed that OM-GF17 scavenged $ABTS^+$, DPPH, NO and decreased iron oxidation. Our results also indicated that F, W, P, C, and M be related to $ABTS^+$, DPPH and NO scavenging activity, as well as the Fe^{3+} reducing power. Furthermore, OM-GF17 did not exhibit direct microbe-killing activity. This novel gene-encoded antioxidant peptide may help in the development of new antioxidant agents and will improve our understanding of the biological functions of amphibian skins.

Disclosure statement

The authors declare no competing commercial interests in relation to this work.

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