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Synergistic effects of antioxidative peptides from rice bran

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Abstract

Generation of excess reactive oxygen species (ROS) such as hydroxyl radicals and hydrogen peroxide results in oxidized cellular environments causing damages to cells. Recently, natural rice bran protein hydrolysates of Khao Dawk Mali 105 rice were reported to possess an antioxidation activity. Several individual peptides were computationally predicted to be responsible for the antioxidation. Here, we aim to confirm and further investigate on the synergistic effect of these predicted peptides using DPPH, ORAC, DNA damage assays, and *in vitro* cellular ROS detection. Three different predicted peptides named FK13, KR10, and CK9 were tested individually and in combination for their antioxidations. In DPPH and ORAC assays, the CK9 peptide individually showed the best scavenging activity with an IC_{50} of 25 µM and an ORAC value of 41 µmole ascorbic acid equivalent/ 15 µmole peptide. For DNA damage assay, CK9 and KR10 synergistically demonstrated the best DNA protection activity at a concentration ratio of 2: 1, respectively. Additionally, a human non-tumorigenic epithelial (MCF-10A) cells, an *in vitro* model, had a significant reduce in the level of ROS after being treated with individual CK9 peptide. In summary, the combination of CK9 and KR10 peptides promotes a synergistic antioxidation potential in DNA protection and can be applied for food and nutraceutical applications to prevent cellular oxidation environments.

Introduction

Reactive oxygen species (ROS), also known as free radicals, are highly reactive molecules containing oxygen atoms such as hydrogen peroxide, peroxyl, hydroxyl radicals. High levels of cellular ROS can trigger an oxidative environment by causing damages to biological molecules inside cells including DNAs, proteins, carbohydrates, and lipids. As a result, a number of antioxidants are used to mitigate and prevent the cellular damages. The antioxidant conceptually inhibits or quenches radical chain reactions. Endogenous antioxidant enzymes convert dangerous oxidative products to hydrogen peroxide (H_2O_2) and then to water, in a multi-step process. Moreover, certain natural compounds exogenously found in plants and animals can act as antioxidants such as vitamin C, vitamin E, polyphenol compounds, and antioxidative peptides.[1] Rice is one of the well-known antioxidant sources from plant. The bran fraction of rice is a nutrient-rich by-product containing good fats and proteins. Rice bran protein hydrolysates have been reported for their biological activities including antioxidation.[2,3] In a recent study, the trypsin-digested albumin fraction from Khao Dawk Mali 105 rice bran protein showed the highest ORAC values.[3] A number of possible amino acid sequences of were also computationally predicted and identified using tandem mass spectrometry (MS/MS).[3] As a result, a further investigation and confirmation of the purified peptides is essential to reveal a relationship between structure, key amino acid residues, and antioxidant mechanisms. Additionally, a database for bioactive peptides from rice bran can be established and used for

improving the efficiency of antioxidants.[4] Furthermore, several studies demonstrated that combinations of antioxidants could synergistically promote their activity to be more potent than a single antioxidant.[5,6] Here, we aim to confirm the antioxidant activities and further investigate on their synergistic effect of those previously predicted peptides using DPPH, ORAC, DNA damage assays, and in vitro cellular ROS detection.

Methodology

Chemicals and peptide synthesis

All chemicals were purchased from Sigma-Aldrich otherwise noted. All peptides were chemically synthesized from GL Biochem (Shanghai) Ltd, China with > 95% purity.

DNA damage assay

A hundred nanogram of DNA plasmid was mixed with 0.1 mM $FeSO_4$ (UNILAB, Australia) and 30% H_2O_2 (Carlo Erba, France) in the presence or absence of peptides. The reaction was incubated at 37ºC for 30 min and then analyzed using 1% agarose gel electrophoresis. The DNA intensity was and calculated by ImageJ for the % DNA protection.

DPPH assay

Different concentrations of individual and mixed peptides were incubated with DPPH at a ratio of 1:20 at room temperature for 30 min in dark followed by a measurement of the absorbance at 517 nm. BHT was used as positive control. The scavenging activity of DPPH radicals was calculated by using the formula below. Finally, the concentration of 50% inhibitory peptide concentration (IC_{50}) was calculated.

$$\% \text{ Scavenging} = (\text{Blank} - \text{Sample}) / \text{Blank} \times 100$$

Oxygen radical absorbance capacity (ORAC)

15 µM of peptides was mixed with 8.16 nM fluorescein in a black 96-well plate. The sample was incubated at 37ºC for 15 min followed by an addition of 153 mM AAPH (EMD, USA). After that, the fluorescence was kinetically measured at excitation 485 nm and emission 535 nm until the reading remained constant. Ascorbic acid was used as a standard with concentration ranging from 6.25 to 100 µM. The ORAC values were calculated by using the following equation. $AUC = 0.5 + f_1/f_0 + f_2/f_0 + \ldots + f_i/f_0 + 0.5\ (f_n/f_0)$

$$\text{Net AUC} = AUC_{sample} - AUC_{blank}$$

f_i and f_0 represents the fluorescence reading at time i and zero, respectively. An ORAC value was calculated using the regression equation between ascorbic concentration and the net AUC and reported as µmole ascorbic acid equivalent/ 15 µmole peptide.

Detection of in vitro cellular reactive oxygen species (ROS)

The intracellular *ROS* generation was evaluated by using carboxy-H_2DCFDA dyes (Thermo, USA). 25,000 cells of normal mammary epithelial cell (MCF-10A) were seeded into 96-well plate and grown overnight. The cells were then stained with 10 µM dye for 2 hours and replaced with 0.25 mM peptides and 0.2 mM H_2O_2 followed by overnight incubation. The level of ROS was analyzed by micro plate reader at Ex/Em: 495/517 nm.

MTT assay

The cells were incubated with 0.5 mg/mL MTT solution (Calbiochem, USA) for 4 hours and replaced with DMSO solution to dissolve water-insoluble purple formazan crystal. The intensity of formazan was measured by micro plate reader at 570 nm with a reference wavelength at 630 nm.

Determination of synergistic potential of the peptide combinations

The synergistic effect of antioxidative peptides was determined by using the difference in antioxidation values between the combination peptides and the sum of the individual peptides ± standard error. Positive values indicated the synergistic antioxidation.

Statistical analysis

Experiments were done in triplicate. $p < 0.05$ was used for statistical test.

Table 1. Sequences of antioxidative peptides*

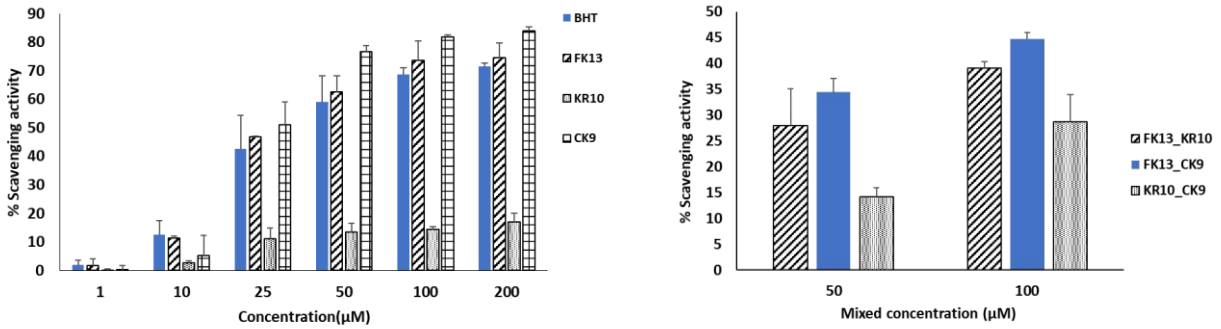
Antioxidative peptides	Amino acid sequence
CK9	CQHHHDQWK
KR10	KEQMGEEGYR
FK13	FIECCSCGEYLKK

* All peptide sequences were computationally predicted and reported elsewhere. [3]

Results and Discussion

Three computationally predicted peptides, named CK9, KR10, and FK13, derived from rice bran, a byproduct of rice milling, of Khao Dawk Mali 105 were examined for their antioxidation synergism. (Table 1.) All peptides consist of amino acids that are known for their intrinsic antioxidant activities including Typ (W), His (H), Cys (C), Lys (K), and Tyr (Y). These amino acids can inhibit the free radicals using aromatic rings, ability to chelate metal ions, and thiol groups.[2,3,7,8] Additionally, these peptides consist of less than 20 amino acids in length which was reported to be optimal lengths for antioxidative peptides.[9] Moreover, either N- or C- termini amino acid has hydrophobicity.

To further investigation on their antioxidation activities, DPPH scavenging activity was first evaluated (Figure 1a). All three individual peptides possessed different degrees of DPPH scavenging activity. However, only CK9 peptide statistically demonstrated higher % scavenging activity than BHT control with the IC_{50} values of 25 and 33 µM, respectively. For a synergistic study, the concentration of individual peptides was fixed at 25 and 50 µM generating a mixed concentration of 50 and 100 µM, respectively (Figure 1b). Compared with individual peptide experiments, all % scavenging activities from peptide combinations were reduced suggesting antagonistic effects. Thus, we concluded that combination of the peptides did not promote the synergistic effect in scavenging DPPH radicals at the concentration tested.



(a) (b)

Figure 1. % Scavenging activity of individual peptides varied from 1 to 200 µM (a) and mixed peptides for synergistic studies (b). The concentration of each peptide was 25 µM and 50 µM, respectively. Butylated hydroxytoluene (BHT) was used as a control.

Next, an oxygen radical absorbance capacity (ORAC) was evaluated at 15 µM peptide concentration (Figure 2). For individual peptides, the ORAC values of FK13, KR10, and CK9 were 27, 38, and 41 µmole ascorbic acid equivalent/15 µmole peptide, respectively. Consistent with DPPH assay, the individual CK9 rendered the best antioxidation activity. The synergistic

effects from 3 peptide combinations including FK13 and KR10, FK13 and CK9, and KR10 and CK9 gave ORAC values of 41, 49, and 34 µmole ascorbic acid equivalent/15 µmole peptide, respectively (Figure 2b). The FK13 and CK9 combination showed highest ORAC values and was more antioxidant than the individual FK13 and CK9 peptides alone. However, its antioxidation activity did not significantly overcome the addition effect which is a sum of ORAC values of each peptide. Varying the ratio of the peptide combinations might be performed to reveal the synergism of the combination.[10] At the concentration tested, the FK13 and CK9 did not synergistically enhance the ORAC values.

Besides, the protection of DNA molecules from hydroxyl radicals was evaluated (Figure 3). Without peptides, the DNA plasmid was completely degraded (Lane 3) compared with the plasmid control (Lane 1) (Figure 3a). After individual peptides were added at 30 µM, only the CK9 peptide partially protected the integrity of DNA (Lane 6). The combination peptides (Lane 7- 9) potentially resulted in the synergistic DNA protection. A combination between FK13 and KR10 peptides (Lane 7) correlated with the results from individual peptides as no DNA protection was observed. On the contrary, the plasmid band's intensity was enhanced in the CK9 and KR10 mixture (Lane 9). The optimization of the peptide ratios was performed by fixing the concentration of CK9 at 90 µM and varying the concentration of KR10 (Figure 3b). The more concentration of KR10, the more intense DNA plasmid bands (upper band) were obtained (Lane 5 - 8). Notably, the synergistic DNA protection activity reached the greatest at 45 µM KR10 (Lane 8) and started to decline at 60 µM (Lane 9). By calculating the band intensity using ImageJ, the concentration ratio of CK9 and KR10 at 90: 45 µM (2: 1) synergistically promoted the DNA protection activity with a synergistic 3.33-fold increase (Figure 3c). Thus, CK9 and KR10 peptides were further explored on inhibiting cellular ROS in normal cells.

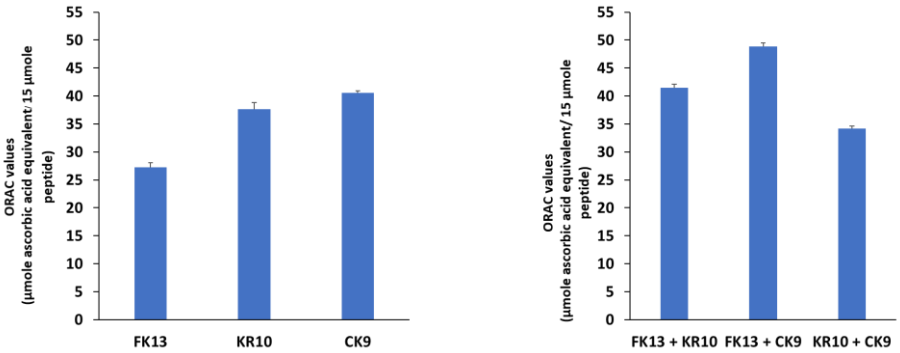


Figure 2. ORAC assays of FK13, KR10, and CK9 peptides at 15 µM (a). ORAC assays of peptide mixture for synergistic effect (15 µM per each peptide) (b).

The inhibition of *in vitro* cellular ROS was examined using a normal human mammary epithelial cell (MCF-10A). Lowering ROS generation in normal human cells is of interest as elevated ROS levels can cause gene mutation leading to many diseases including cancers. 250 µM of both peptides alone were non-toxic to the cells (data not shown). A cellular oxidative stress was induced with 0.2 mM H_2O_2 by which the cell viability was not statistically compromised (Figure 4a). The fluorescence intensity generated by carboxy-H2DCFDA dye represented the level of ROS generated inside the cells (Figure 4b). The level of cellular ROS was significantly decreased by approximately 1.4 folds in 0.25 mM CK9 peptide treatment. The combination of CK9 and KR10 was likely to be synergistic in reducing the level of ROS generated inside MCF-10A cells. However, further experiments are required to improve a statistical reliability at $p < 0.05$.

Last but not least, the interactions between synergistic or antagonistic peptides and their relationship to the mechanism of action still remain unclear. Also, it is possible that the combination of all three peptides may give even more synergistic antioxidation. More studies should be further conducted to provide an understanding in the synergistic mechanisms, maximize the use, and avoid adverse effects from the peptide combination from rice bran.

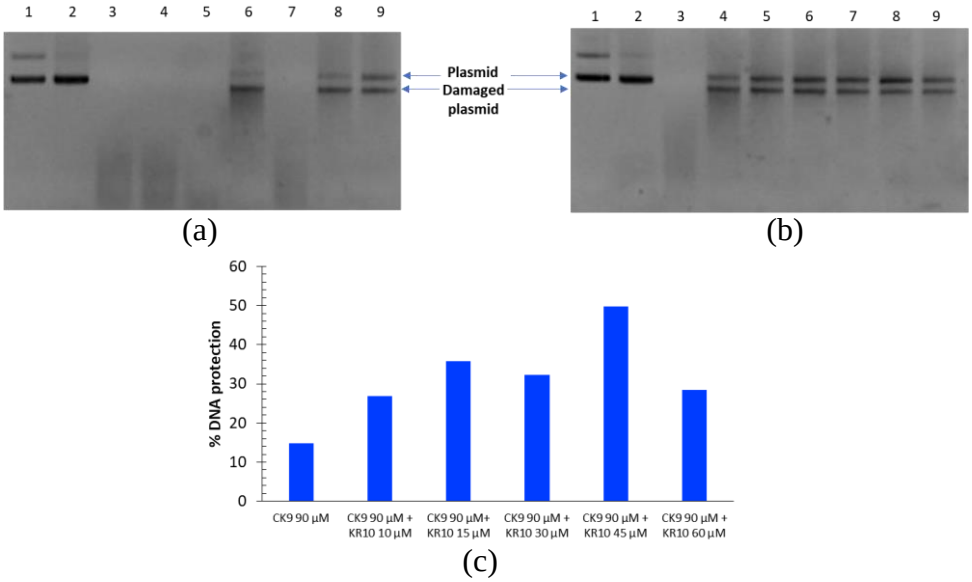


Figure 3. DNA damage assay of individual and mixed peptides at 30 µM (a). Optimization of synergistic DNA damage assay between CK9 (90 µM) and KR10 (10, 15, 30, 45, and 60 µM) (b). Quantification of % DNA protection of the combination of CK9 and KR10 at different concentrations (c). For image (a): Lane 1. Plasmid control, 2. Plasmid + $FeSO_4$ control, 3. Plasmid + $FeSO_4$ + H_2O_2 control, 4. FK13, 5. KR10, 6. CK9, 7. FK13 + KR10, 8. FK13 + CK9, 9. CK9 + KR10. For image (b): Lane 1. Plasmid control, 2. Plasmid + $FeSO_4$ control, 3. Plasmid + $FeSO_4$ + H_2O_2 control, 4. CK9, 5.-9. CK9 + KR10 (10, 15, 30, 45, and 60 µM, respectively)

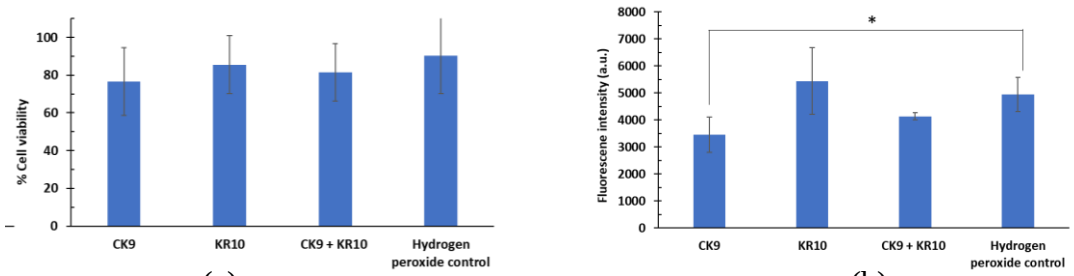


Figure 4. Cell viability (a) and levels of *in vitro* cellular ROS (b) of MCF-10A cells during oxidative stress induction by 0.2 mM H_2O_2. (* $p < 0.05$)

Conclusion

The individual CK9 peptide ranked the highest in antioxidant activities. Moreover, we proposed, for the first time, the synergistic action of antioxidative CK9 and KR10 peptide combination obtained from rice bran. The CK9 and KR10 peptides can also be used as model peptides to search for the same antioxidative peptides in other natural products or further used in many food and biological applications.

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