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Potential Antioxidant Peptides in Rice Wine

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Abstract Many food protein hydrolysates have been shown to have antioxidant activities, and recent research focuses on low molecular peptides produced during hydrolysis of food protein. Korean rice wine contains about 60–70% of protein at dry base and originates from raw materials. It has been suggested that the protein is transformed into low molecular weight peptides, and have antioxidant activity during fermentation. The objectives of this study were to evaluate the antioxidant activity of the pre-purified and purified peptides found in Korean rice wine and to identify the responsible peptides. The wine extract of *Samhaeju*, a traditional Korean rice wine made by low temperature fermentation, was evaporated at 35°C. The two methods employed in the evaluation of antioxidant activity were the DPPH radical scavenging method and the beta-carotene bleaching test. The pre-purified samples showed 808 AAC (Antioxidant Activity Coefficient) and 56.5% AOA (Antioxidant Activity), which were higher than α-tocopherol (572 AAC and 78% AOA). The rice wine extract was separated by reversed-phase HPLC. The protective effect of the four most antioxidant active fractions were tested for t-butyl hydroperoxide induced oxidation of healthy human erythrocytes and the byproduct was determined by malondialdehyde formation. Fraction No. 5 showed 35% lower MDA concentration as compared to the control. The peptides were further purified using consecutive chromatographic methods and 4 antioxidant peptides were isolated. The amino acid sequences of the peptides were identified as Ile-His-His, Val-Val-His(Asn), Leu-Val-Pro, and Leu(Val)-Lys-Arg-Pro. The AAC value of the synthetic form of the identified peptides was the highest for Ile-His-His.

Key words: Rice-wine, antioxidant peptide

Food protein, particularly the hydrolysate type, has been considered to be a health-benefiting functional food. In particular, the peptides with N-terminal leucine/valine of beta conglycinin found in soybean [6], histidine in the second residue of 3 types of peptide in white egg albumin [35], and hydrolysate of bovine skin [19] have all been shown to have antioxidant properties. In food, the safety problem of synthetic antioxidants like BHA and BHT needs to be resolved. Therefore, the application of natural antioxidants such as peptides in food and other health products are important, and research is currently being carried out by scientists to elucidate these antioxidant peptides [3, 12]. *In vivo* studies have shown that peptides from soybean increased the immune response of rats, and a peptide extracted from Japanese rice wine, *Sake*, had an antihypertensive effect on spontaneous hypertensive rat (SHR) [39]. Furthermore, it has been suggested that active peptides in functional foods have low molecular weight and are composed of 3–16 amino acids [6, 19, 35].

Although much of the functional peptide research has been oriented toward hydrolysates of food protein, more and more is being focused on other foods. For instance, a Japanese group has identified the structure of angiotensin converting enzyme (ACE) inhibition peptide in *Sake* [39, 40]. In Europe, the consumption of red wine is favorably regarded, as it has been claimed to be health promoting due to high polyphenol content [1, 20]. Likewise, in many Asian countries, consumption of rice wine is common. In Korea, a variety of rice wine is found and *Chongju* for example is regularly taken with meals and has been traditionally alleged to maintain health [15, 25], but scientific evidence is scarce. The fine form of *Chongju* (*Samhaeju*) is made by fermentation at a low temperature, and excels in protein content compared to other alcoholic beverages such as wine and liquors.

In order to understand the rationale of *Samhaeju* in the aspect of their health benefits and long shelf-life, we have assessed the antioxidant property of the peptides produced during fermentation of traditionally made *Samhaeju* and identified the amino acid sequences for the responsible antioxidant peptide.

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MATERIALS AND METHODS

Preparation of Rice Wine

Traditional rice wine (*Samhaeju)* processing adapts low temperature (5-10°C), long-time fermentation for over three months using rice, *nuruk*, the fermentation starter [26] and water according to Rhee's method [27, 30].

Preparation of Rice Wine Sample

The rice wine was prepared according to Rhee *et al.* [30]. The alcohol portion of the rice wine was removed by adding 50 ml of rice wine to 50 ml of distilled water and concentrated to the original volume under vacuum using a rotary evaporator at 35°C.

Free Radical Scavenging Activity

The overall antioxidant activity of the prepared sample was assessed by the DPPH method [21, 22]. DPPH is a stable free radical that loses its absorbance at 517 nm when it is reduced. DPPH stock was prepared by dissolving 16 mg of DPPH in 100 ml absolute ethanol. A volume of 50 µl wine sample was added to 0.5 ml of DPPH solution and made up to 1 ml with absolute ethanol. The mixture was shaken vigorously and the absorbance was measured at 517 nm with a spectrophotometer for 30 min. As positive control, α-tocopherol (500 µM) was used. The percentage of inhibition, which represents the scavenging ability of the sample on DPPH radical, was calculated as follows [22]:

Antioxidant Activity (AOA)
=100-[(absorbance increase of sample/
absorbance increase of control)×100]

Lipid Peroxidation

The antioxidant activity against oxidation of lipid by the samples was analyzed by the beta-carotene bleaching test (BCBT) [22] and oxidation of erythrocytes [10]. To an aliquot of 1 mg β-carotene (0.2%) disolved in chloroform, 25 µl linoleic acid and 200 mg Tween 40 were added. The chloroform part was evaporated under vacuum at 40°C and 50 ml of oxygenated ultra-pure water was added. The mixture was vigorously shaken and aliquots of 250 µl of the emulsion were placed in 96-well microtitre plate and 30 µl of the sample was added. The microtitre plates were incubated at 55°C for 105 min, and the absorbance was measured at 492 nm (EAR 400 Microtitre, U.S.A.). The antioxidant activity of the sample against lipid oxidation is expressed as Antioxidant Activity Coefficient (AAC) in which,

$$AAC=[(A_{A,105}-A_{B,105}/A_{B,0}-A_{B,105})]\times 1{,}000$$

$A_{A,105}$ and $A_{B,105}$ are the absorbances of the test and blank sample, respectively, at t=105 min, and $A_{B,0}$ is the absorbance of the blank sample at t=0 min [22].

A fasted blood sample (3 ml) taken from a volunteer was collected in EDTA tubes for the erythrocyte oxidation test. The blood was centrifuged at 400 ×*g* for 10 min and the supernatant was removed and the red blood cells were washed three times with PBS (pH 7.3) [10, 33]. In a 50 ml flask, 0.2 ml of rice wine sample, 0.2 ml of 2 mM-t-butylhydroperoxide (t-BHP), and 0.2 ml of 20% (v/v) erythrocyte suspension prepared in PBS were made up to 4 ml with KRP buffer (120 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 16.5 mM NaH_2PO_4/Na_2HPO_4, pH 7.4). The flasks were capped and incubated for 2 h at 37°C in the dark [13, 34].

The degree of lipid peroxidation was determined by measuring the reaction of thiobarbituric acid (TBA) to form malondialdehyde (MDA). After treatment, the erythrocytes were centrifuged (400 ×*g*, 10 min). To 2 ml of the supernatant, 1 ml of 30% trichloroacetic acid was added, mixed, and then centrifuged at 5,000 ×*g* for 15 min. A volume of 0.5 ml TBA (1% w/v) prepared in 0.05 M NaOH was added to 2 ml of the supernatant and then heated in boiling water bath for 10 min and cooled. Using a spectrophotometer, the absorbance was determined at 532 nm [13]. The concentration of MDA was calculated by molar coefficient (1.56×10^5 cm^{-1} path length) and expressed as per ml erythrocyte [10].

Sample Purification

The prepared sample was fractionated on Superdex Peptide HR 10/30 (10×300 mm, Pharmacia, U.S.A.) column with 50 mM ammonium acetate buffer. The GPC was performed on HPLC connected to a UV detector, which was set at 214 nm and 280 nm (Gilson, U.S.A.) and at a flow rate of 0.3 ml/min. The standards used for the molecular weight distribution were lysozyme (14,300, R.T. 34 min), aprotinin (6,500, R.T. 37 min), insulin chain B (3,459, R.T. 39 min), substance P (1348, R.T. 53 min), glutathione (307, R.T. 57 min), and glycine (75, R.T. 61 min) [18].

The sample separation was carried out using a HPLC method by prep liquid chromatography with a RP C18 column (21.2×250 mm, Zorbax RX-C18, Hewlett Packard, U.S.A.). The flow rate was adjusted to 15 ml/min and 5 ml of the sample pre-filtered through a 0.25 µm PTFE membrane was injected. The eluent A (0.1% TFA in distilled water) and B (0.086 TFA in 80% ACN) were also filtered before use. The gradient of eluent B increased from 0 to 60% over 60 min and then further increased to 100%, and maintained for 3 min [18].

The fractionated samples from prep-HPLC showing high antioxidant activity was dissolved in distilled water and loaded on C18 column (3.9×300 mm, Nova-Pak C18, Waters, U.S.A.), which was previously equilibrated with 0.1% TFA in water. The column was then eluted with the same buffer, and eluted with a gradient of 5% of 0.086%

TFA in 80% ACN. The fractions showing antioxidant activity were pooled and lyophilized. These fractions were eluted and further purified by consecutive chromatographic methods using different gradient conditions [23].

Identification of Antioxidant Peptides

Edman degradation using an automated protein sequencer equipped with an HPLC system (Procise clc 492 protein sequencer, Applied Biosystems, U.S.A.) was used for the analysis of the amino acid sequence of antioxidant peptides [14, 28, 29].

Synthetic Peptide Synthesis

The peptides with active antioxidant properties were synthesized according to the sequence identified by the Fmoc solid-phase method (Peptron Inc., Daejoen, Korea) [16, 18].

Statistical Analysis

All values are expressed as the mean±SD. Tests for the statistical significance of differences were compared by Student's *t*-test for all experiments.

RESULTS AND DISCUSSION

Antioxidant Activity and Molecular Size of the Substances in Rice Wine Extract

The key functional groups involved in the biochemistry of red wine have been identified to be polyphenols. However, investigations on the health benefiting function of rice wine in Korea are based on crude extract and many results failed to prominently identify the active biochemical componds. Table 1 shows the chemical composition and total polyphenol content of *Samhaeju*. Compared to red wine (2,000 ppm), the level of total polyphenol in *Samhaeju* is very low (203 ppm). On the other hand, the protein content of *Samhaeju* (1.66%) is much higher than that in red wine (0.2%) [7].

Rice wine contains approximately 70% protein in dry base. During fermentation, the raw materials, which are rich in protein, transform into low molecular weight peptides [26]. We postulate that the antioxidant activity in rice wine is due to these peptides because antioxidant vitamins or other micronutrients levels are extremely low [7]. Apart from the antioxidant property, peptides found in rice wines such as Japanese *Sake* showed ACE inhibition [39, 40]. Recently, Kim *et al.* [17] and Lee *et al.* [24] found that the health benefit was increased when the rice wine was fermented and suggested that the functional group responsible for this effect was peptides. In this study, the authors also agree with the previous reports, in which the peptides formed during the fermentation of rice wine has a strong health beneficial functional property.

In order to study the antioxidant effects of rice wine, we used two widely known methods, namely, DPPH and BCBT. There are many methods to evaluate antioxidant activity, however the strengths and limitations of each method depend on the number of samples and sample polarity. The DPPH method is convenient and quick for screening many samples and is independent from sample polarity. The results of the pre-purified rice wine sample showed 56.5% AOA and 808 AAC, whereas the positive control, 500 μM α-tocopherol, showed 77.1% AOA and 572 AAC (Table 1).

The molecular size distribution of rice wine showed three major peaks with less than 1.2 kDa and had relatively potent AOA activity (data not presented). The result indicates that the peptides have considerable low molecular weight and are composed of less than 12 amino acids.

Chen *et al.* [6] also showed that soybean beta conglycinin contains antioxidant peptides and is composed of 5 to 16 amino acids. In other reports, Kim *et al.* [19] found that the hydrolysate of bovine skin had antioxidant peptides composed of 9 to 10 amino acids, and Tsuge *et al.* [35] found antioxidant peptide in egg white albumin, which consists of 2 to 7 amino acids. In earlier findings, peptides from hydrolysates of gelatin, soy protein, milk casein, and egg white albumin with molecular weight approximately 2.0 kDa had high antioxidant activity [36, 37].

Table 1. Chemical composition and total polyphenol content of *Samhaeju*.

Moisture	84.0%
Alcohol	17.0%
Protein	1.6%
Carbohydrate	0.7%
Ash	0.1%
Fat	0%
Total phenolic compounds	203 ppm

Table 2. Antioxidant activity of rice wine fractionated by prep HPLC.

Fraction	AOA	AAC
F3	7.3±0.6	343.1±21.0
F4	31.5±4.8	472.73±5.5
F5	43.6±2.5	405.9±46.7
F10	46.8±4.2	317.9±11.3
F11	32.3±2.4	312.6±10.5
F16	15.8±0.7	328.4±36.2
F19	15.2±1.0	468.1±57.6
F31	32.93±2.6	72.7±0.1
F33	32.5±1.2	59.2±2.5
Original rice wine	56.5±4.69	808±9.85
Vit E (500 μM)	77.1±2.2	572±2.7

AOA: Antioxidant activity as determined by DPPH method.
AAC: Antioxidant activity coefficient as determined by BCBT method.

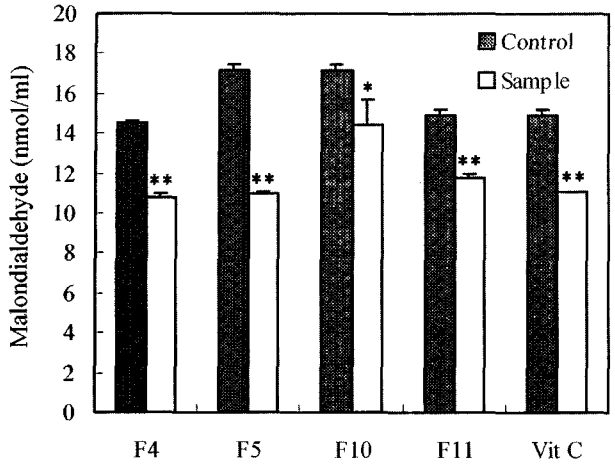

Fig. 1. Antioxidant effect of fractionated sample against erythrocyte oxidation.
*P<0.05 control vs sample, **P<0.01 control vs sample.

Table 3. Antioxidant activity of rice wine fractionated by HPLC.

Fraction	AAC
F5-3	364±23.2
F5-4	47±2.56
F5-5	82±6.35
F5-6	152±11.9
F5-7	423±38.6
F5-8	211±18.7
F5-9	11±1.3
F5-10	82±7.9
F5-11	435±42.1
F5-12	247±11.2

AAC: Antioxidant activity coefficient as determined by BCBT method.

Antioxidant Activity of Purified Peptides

Sixty fractions of rice wine concentrate separated by prep liquid chromatography (Table 2) were evaluated for antioxidant activity. Of the fractions, 6 (F4, F5, F10, F11, F31, F33) had AOA values over 30%, and 7 fractions (F3, F4, F5, F10, F11, F16, F19) had AAC values over 300, demonstrating the high antioxidant activity of the fractions.

Of the sample, the four fractions which had the highest antioxidant activity in each test were analyzed for their protective effect against t-BHP induced oxidation of healthy human erythrocytes, which was determined by the MDA formation. The erythrocyte used in this investigation was collected from one person in order to minimize

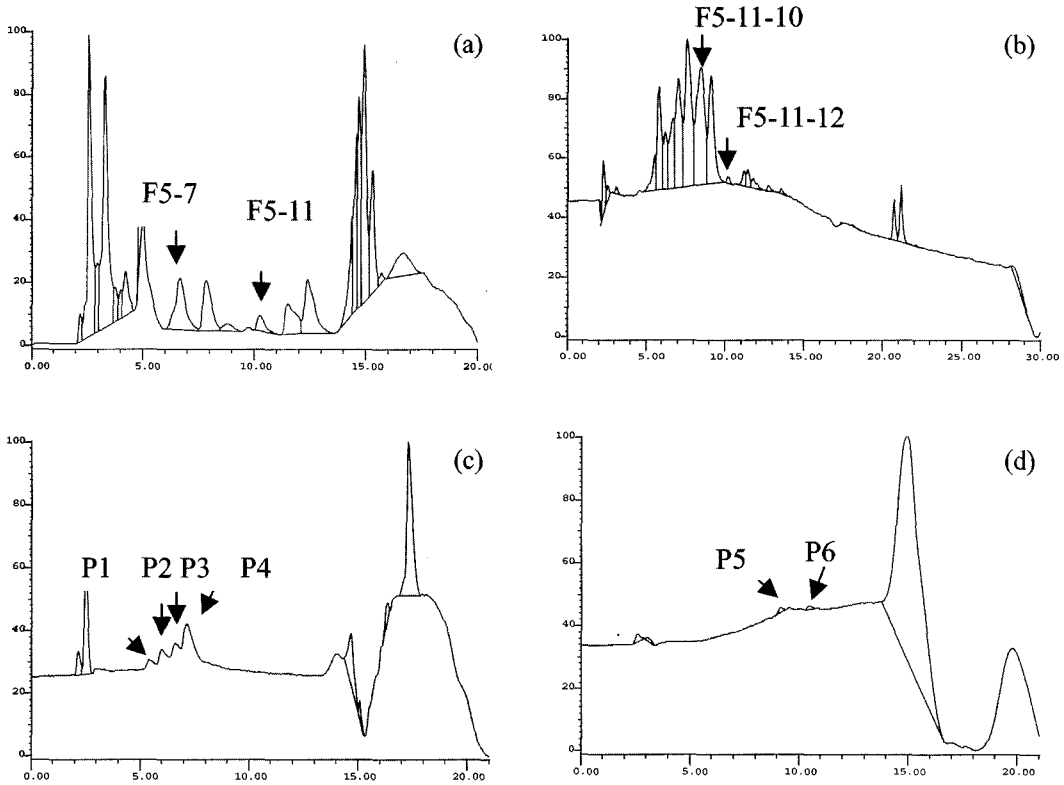

Fig. 2. Purification of antioxidant peptides from rice wine.
The sample fractions were collected at the retention time presented in the chromatogram. Fractions showing antioxidant activity were indicated by an arrow. Figures represent (a) the separated fraction of sample F5, (b) the separated fraction of sample F5-11, (c) the separated fraction of sample F5-11-10, and (d) the separated fraction of sample F5-11-12.

individual variance. The amount of MDA formed from t-BHP-induced erythrocytes was extremely high but was inhibited by rice wine extract. The inhibition rate of the fractionated samples ranged from 15.6% to 35.8%. Fraction number 5 (F5) showed the greatest inhibition rate and was comparable to the positive control, 75 μmol ascorbic acid (25.8%) (Fig. 1). This inhibition is greater than that found by Tedesco *et al.* [33] on red wine extract, which had an inhibition rate of 28% as compared to the control. Since F5 showed high antioxidant activity, it was further separated by reverse-phase liquid chromatography. AAC was widely observed for F5-3 to F5-12 fractions; in particular, F5-7 and F5-11 were 435 and 423, respectively, which surpassed the other fractions (Table 3).

In order to elucidate the peptide responsible for the antioxidant activity, further purification was made by HPLC for the sample fraction with the highest antioxidant activity (F5-7 and F5-11) (Fig. 2). Two of the fractions found, F5-11-10 and F-5-11-12, showed high AAC, in which the value (153) was similar.

Identification of the Purified Peptide

As the 2 fractions (F5-11-10 and F-5-11-12) of the highest AAC value were not pure enough for the elucidation of the peptide, it was further separated by HPLC under different conditions. Four fractions (P1, P2, P3, P4) were obtained from F5-11-10 and 2 (P5, P6) from F5-11-12 (Fig. 2). Overall, the peptide obtained from the fractions were very short and was found to be composed of approximately 3 to 4 amino acids. The sequences of P3 and P4 were identified as Val-Val-His(Asn) and Ile-His-His, respectively, and Leu(Val)-Lys-Arg-Pro and Leu-Val-Pro for P5 and P6, respectively (Table 4). However, the sequences of P1 and P2 were indistinct.

In the sequences of P3 and P4, hydrophobic amino acids, valine and isoleucine, were found in the N-terminal, and histidine, which has been claimed to be a radical scavenger, was found in the C-terminal. Likewise, the hydrophobic amino acids leucine and valine were found in the N-terminal, and radical scavenging proline was found in the C-terminal of P5 and P6. Chen *et al.* [6] found that the antioxidant peptides from soybean beta-conglycinin were composed of hydrophobic amino acid at the N terminus, including valine or leucine, and proline, histidine, and tyrosine in the sequences. Also, Tsuge *et al.* [35] isolated 3 peptides from egg white albumin with antioxidant activity, in which histidine was found in the second residue of all 3 peptides (Ala-His, Val-His-His, Val-His-His-Ala-Asn-Glu-Asn).

Table 4. Amino acid sequences of isolated antioxidant peptides from rice wine.

Fraction	Sequence
p1	Not identified
p2	Not identified
p3	Val-Val-His(Asn)
p4	Ile-His-His
p5	Leu(Val)-Lys-Arg-Pro
p6	Leu-Val-Pro

Several amino acids, including histidine, proline, methionine, tyrosine, have been recognized to show antioxidant activity. In general, peptides showed higher antioxidant activity than those of single amino acid mixtures and this was partly explained by the increase of hydrophobicity in the peptides, which leads to a higher interaction between the peptide and fatty acids [4, 5]. Uchida and Kawakishi [36] have identified the antioxidant activity of histidine-containing peptides. The antioxidant activity of histidine-containing peptides is attributed to the chelating and radical-trapping abilities of the imidazole ring. Moreover, in the study of antioxidant peptide from soybean protein, LLPHH, Chen *et al.* [5] showed that the deletion of N-terminal leucine of LLPHH did not affect the antioxidant activity, but deletion of C-terminal His caused loss of activity.

Also, as in the structure of P3 and P4, the hydrophobic amino acids, valine and leucine, were found in the N-terminal of P5 and P6, and proline is also claimed to be a radical scavenging amino acid found in the C-terminal. Hydrolysates of protein from Alaska Pollack skin and bovine skin gelatin are rich in proline and glycine. The secondary amine structure of proline forms a stable nitroxide radical in the presence of foreign radicals [5]. Suetsuna [32] showed that antioxidant peptides found in prawn muscle are composed of 3 to 4 amino acids and identified as hydrophobic phenylalanine and isoleucine in the N-terminal and lysine (Ile-Lys-Lys, Phe-Lys-Lys, Phe-Ile-Lys-Lys).

In this study, the strong antioxidant property of the peptide may be accredited by the short chain and very hydrophobic amino acid (Ile and Val) at the N-terminal in conjunction with antioxidant histidine and proline in the sequence. However, the significance of these peptides in the biological system is unknown. Many of the active peptides may lose their activity because they are hydrolyzed with protease in the digestive system, or may not even be digested or absorbed when administered orally. However, *in vivo*, some di- or tri-peptides can be absorbed directly and the process is more rapid than absorption of an equivalent mixture of free amino acids [11]. Peptide transport, in which two-thirds of the amino acids are absorbed in the form of small peptides, with the remaining 1/3 absorbed as free amino acids [8, 9, 31], represents the primary system for amino acid absorption. Thus, it may prove the old belief that drinking rice wine moderately and regularly extends the longevity of life, and exerts an anti-ageing effect [2].

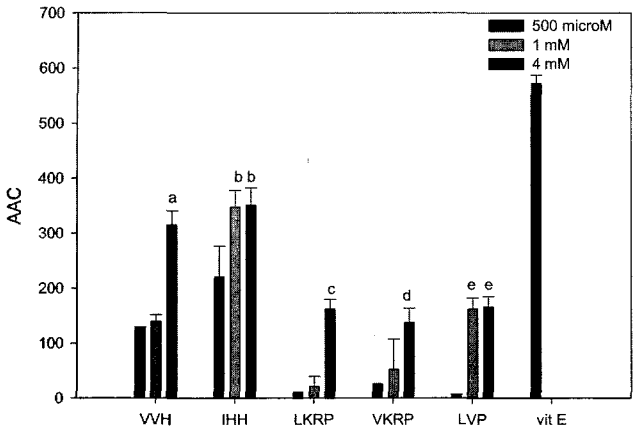

Fig. 3. Antioxidant activity of synthetic peptides.
a, $p<0.01$ 4 mM vs 500 μM, 1 mM; b, $p<0.01$ 500 μM vs 1 mM, 4 mM; c, $p<0.01$ 4 mM vs 500 μM, 1 mM; d, $p<0.01$ 4 mM vs 500 μM, 1 mM; e, $p<0.01$ 500 μM vs 1 mM, 4 mM.

Antioxidant Activity of Synthetic Peptides

In order to confirm the findings of the sequences of the peptides responsible for the high antioxidant activity, the peptides were synthesized and re-tested by the BCBT method (Fig. 3). Apart from Val-Val-Asp (VVN), all the synthetic peptides had antioxidant activity. The concentrations of the peptides tested were 500 μmol and 4 mM, and peptides that include His (Val-Val-His and Ile-His-His) showed the highest AAC value but were not dose-dependent. On the other hand, the antioxidant effect of Leu-Lys-Arg-Pro(LKRP) and Val-Lys-Arg-Pro(VKRP), in which the sequences are the same except at the N-terminal, was affected by the dose. Shorter peptides, which include hydrophobic proline (Leu-Val-Pro), had lower antioxidant activity than LKRP and VKRP. The ability of the synthetic peptides to scavenge radicals formed from t-BHP-induced erythrocytes was weak and the most effective peptide was VKLP (Fig. 4).

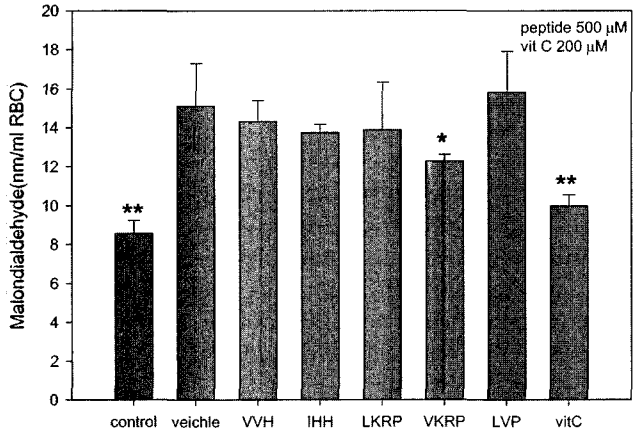

Fig. 4. Antioxidant effect of synthetic peptides against erythrocyte oxidation.
*$p<0.05$ vehicle vs VKRP, **$p<0.01$ vehicle vs vit C, control vs vehicle.

In general, the findings from this study indicate that like antioxidant-rich red wine, which is health-promoting when consumed in moderate amount, rice wine has the potential to have a similar effect.

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