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RESEARCH NOTE

# Antioxidative and Nitric Oxide Scavenging Activity of Branched-Chain Amino Acids

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**Abstract** The purpose of this study was to determine the inhibitory effect of branched-chain amino acids (BCAAs: leucine, valine, and isoleucine) on lipid peroxidation, and their nitric oxide (NO) scavenging activity. The inhibitory effect of BCAAs on lipid peroxidation was measured by the ferric thiocyanate (FTC) method, and their NO scavenging activity was evaluated using the sodium nitroprusside (SNP) assay. The FTC method results indicated that valine was the most effective of the BCAAs, with 100 mM of valine showing 72.05% antioxidative activity. An SNP assay conducted for 24 h produced the least amount of NO (98.52 μM) when it included 100 mM leucine compared to the other BCAAs (control: 132.20 μM). The results of this study suggested that BCAAs can be used to develop antioxidant or antiinflammatory products in the food or pharmaceutical industries.

**Keywords:** branched-chain amino acid, antioxidative effect, nitric oxide scavenging effect, ferric thiocyanate method, lipid peroxidation

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## Introduction

Oxidative stress is defined as the imbalance between oxidation and anti-oxidation in living cells (1) and is caused by the peroxidation of various cellular lipids, proteins, carbohydrates, and nucleic acids. In particular, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are highly reactive due to their unpaired electrons, and both of them cause oxidative stress as well as nitrosative stress in biological systems (2). Therefore, biological oxidation results from an imbalance between the production of ROS/RNS and their removal by available antioxidant systems (1). It has been known that biological oxidation damages multiple types of biological substances and causes many diseases. Recently, there have been many studies on the relationships between these stresses and various diseases, including cancer, aging, and inflammation (3). As a result, many interested researchers have turned their attention to preventing oxidative and nitrosative damage (3). A few amino acids have been known to have antioxidative effect. For example, Atmaca (4) reported that sulfur-containing amino acids, such as cysteine, methionine, and taurine, have antioxidant effect by mentioning that the number of sulfur atoms and the oxidation levels of sulfur atoms in sulfur-containing compounds can affect, at least in part, their antioxidant activity. Wade and Tucker (5) reported that L-histidine may work as significant physiological antioxidant defenses.

The branched-chain amino acids (BCAAs), which include leucine, valine, and isoleucine, are three of the nine essential amino acids for humans, which need to be consumed as nutrients. They account for approximately

one-third of the essential amino acids in muscle proteins and nearly 40% of the preformed amino acids required by mammals (6). These amino acids have unique chemical structures, including aliphatic side chains with a branch, which is why the BCAAs are named so (7). The functions of BCAAs have been studied extensively. That is, BCAAs affect gene expression, protein metabolism, apoptosis, insulin resistance (8), and are involved in several important brain functions (7). Therefore, BCAAs have the potential for new uses as biofunctional natural resources. Ichikawa *et al*. (9) reported that BCAA-enriched nutrients stimulate antioxidant DNA repair in a rat model of liver injury induced by carbon tetrachloride, but there have been little studies on antioxidative activity of BCAA only. As mentioned earlier, oxidative stress is a major cause of many kinds of diseases (particularly, adult diseases) in humans. However, although recent studies have revealed various functions of BCAAs, little is known about their inhibitory effect on lipid peroxidation or their nitric oxide (NO) radical scavenging activity as biofunctional activities.

Therefore, the objective of this study was to measure BCAA inhibition of lipid peroxidation using a ferric thiocyanate (FTC) assay to detect the initial stage of lipid peroxidation. In addition, NO scavenging activity was evaluated with a sodium nitroprusside (SNP) assay.

## Materials and Methods

**Chemicals** BCAAs (leucine, valine, and isoleucine) were purchased from Cremar Co. (Seongnam, Korea). Linoleic acid, ascorbic acid, sodium nitroprusside (SNP), N-(1-naphthyl)-ethylene diamine hydrochloride, and sulfanilamide were purchased from Sigma Aldrich (St. Louis, MO, USA). Potassium phosphate monobasic ($KH_2PO_4$), potassium phosphate dibasic ($K_2HPO_4$), phosphoric acid ($H_3PO_4$), and ferrous chloride ($FeCl_2$) were procured from Samchun Pure Chemical (Seoul, Korea). Ammonium thiocyanate ($NH_4SCN$) was obtained from Junsei Chemical Co. (Tokyo, Japan). All other chemicals and reagents used in this study were of analytical grade.

**Ferric thiocyanate assay** The inhibitory activity of BCAAs against lipid peroxidation was evaluated using a modification of the method of Lee *et al.* (10) and Jung *et al.* (11). BCAAs were dissolved in distilled water at different concentrations, and 100 μL of the solution was added to a mixture of 400 μL of 40 mM potassium phosphate buffer (pH 7.0), 200 μL of distilled water, and 200 μL of 25 mg/mL linoleic acid in ethanol, to prepare the reaction mixture. Antioxidative activity on linoleic acid was measured after 0, 24, 48, and 72 h during incubation at 37°C in the dark. A 100 μL sample of the reacted mixture was added to 4 mL of 70% ethanol, and then 100 μL of 30% ammonium thiocyanate and 100 μL of 20 mM $FeCl_3$ in 3.5% HCl were added. The red color development that represents the oxidation of linoleic acid was measured at 500 nm with a spectrophotometer (2120UV; Optizen, Daejon, Korea). A 100 mM ascorbic acid solution was used as the positive control. All tests were performed in triplicate.

**NO scavenging assays** NO was generated from SNP and measured by the Griess reaction with slight modifications (12). To make an aqueous solution of SNP, 10 mM SNP was dissolved in 0.1 M phosphate buffer (pH 7.4). To test the samples, 1 mL of sample dissolved in 0.1 M phosphate buffer was added to a test tubes containing 1.0 mL of SNP solution (10 mM), then incubated at 37°C in a water bath for 24 h. Samples were stirred with a vortex mixer (G-560; Scientific Industries, New York, NY, USA) after incubation and 100 μL of samples were added to 100 μL of Griess reagent (a mixture of 1% sulfanilamide in 5% phosphoric acid, and 0.1% N-(1-naphthyl)-ethylene diamine hydrochloride in distilled water) in a 96-well microplate. The absorbance of samples was measured after 15 min at 540 nm with a microplate reader (Emax E-11788; Molecular Devices, Sunnyvale, CA, USA).

**Statistical analysis** Each experiment was performed in triplicate. Analysis of variance (ANOVA) was performed using SPSS 18 (SPSS Inc., Chicago, IL, USA), and a significance difference was defined as $p<0.05$. Significant differences between mean values were determined by one-way ANOVA followed by Duncan's multiple range test.

## Results and Discussion

**Inhibition of lipid peroxidation of BCAAs** Antioxidative activity of BCAAs was determined using the FTC assay (11), which measures the amount of peroxide produced at the initial stage of lipid peroxidation. During oxidation of linoleic acid, peroxide is formed and ferrous ions ($Fe^{2+}$) are oxidized to ferric ions ($Fe^{3+}$). The ferric ions form red colored complexes with ammonium thiocyanate; therefore, a lower absorbance indicates a higher level of antioxidative activity. The change in absorbance for each fraction during 72 h of incubation at 37°C in the dark was shown in Table 1. Leucine, valine, and isoleucine at 100 mM showed the greatest antioxidative activity, and the results among the samples were not significantly different ($p>0.05$). Particularly, 100 mM valine showed 72.05% antioxidative activity, which was higher than leucine and isoleucine, which were 69.97 and 71.67%, respectively, at same concentration. BCAAs at 25 mM were significantly different from the BCAAs at 50 and 100 mM. All of the BCAA samples had

**Table 1. Inhibitory effect of branched-chain amino acids (BCAAs) on lipid peroxidation after a 72 h reaction, as determined by the FTC assay**

| BCAA | Concentration (mM) | Antioxidative activity (%)[1)] |
|---|---|---|
| Leucine | 25 | 56.54±5.029[d] |
| | 50 | 70.13±2.726[b] |
| | 100 | 69.97±3.313[b] |
| Valine | 25 | 51.51±3.844[d] |
| | 50 | 68.93±4.131[b] |
| | 100 | 72.05±1.996[b] |
| Isoleucine | 25 | 59.63±2.314[c] |
| | 50 | 68.01±4.582[b] |
| | 100 | 71.67±3.084[b] |
| Ascorbic acid | 100 | 87.80±5.361[a] |

[1)]Values are mean±SD. Values followed by different letters within the sample type are significantly different ($p$<0.05), while values followed by the same letter are not significantly different. Data were grouped by Duncan's multiple range test.

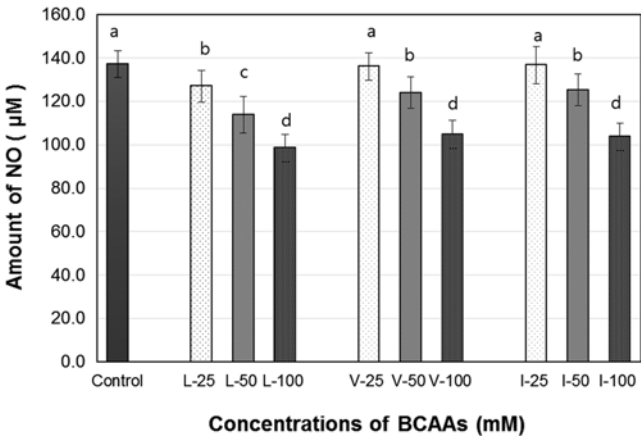


**Fig. 1. Inhibition of nitrite production by BCAAs in a SNP system.** Control reaction shows basal NO production in distilled water induced by SNP. The effect of 25, 50, and 100 mM of BCAAs against NO production was determined by the Griess method. L, leucine; V, valine; I, isoleucine; All values presented as a mean±SD. Bars labeled with different letters indicate significant differences ($p$<0.05) based on a one-way ANOVA, while bars labeled with the same letter are not significantly different.

lower antioxidative activity than ascorbic acid (the positive control). However, according to Lee *et al*. (13), ascorbic acid is very sensitive to light, to the action of oxidizing agents and metal ions, and even to slight heating. Additionally, ascorbic acid levels decline during long-term food storage and processing (14). The results indicated that BCAAs have an inhibitory effect against linoleic acid peroxidation and may potentially be used as new stable antioxidative materials for the food industry. Antioxidants from natural sources could also be a good alternative to synthetic antioxidants in counteracting oxidative stress associated diseases (15).

**NO scavenging activity of BCAAs** NO is a multi-functional gaseous molecule and a highly reactive free radical (16). NO has been regarded as an important biological factor, as it is a fundamental molecule studied in the fields of neuroscience, physiology, and immunology (17). This molecule is involved in the regulation of blood flow in different parts of the body, regulation of blood pressure, prevention of platelet aggregation, adhesion of platelets, and assisting the immune system to kill a wide variety of pathogens (18). Although NO is considered only mildly reactive, it is able to rapidly react with oxygen to form the much more powerful oxidant peroxynitrite (18). These molecules have the potential to produce toxic reactions, such as lipid peroxidation, and to induce cell death (19). Additionally, NO is considered a signaling molecule that plays a key role in inflammation (20). Reactive nitrogen intermediates, such as NO, peroxynitrite, and nitrogen dioxide, have been shown to be key factors in the inflammatory process (19). In this study, the antioxidative potency through NO scavenging of the BCAAs was evaluated spectrometrically (21). The differences in the NO scavenging effects of different concentrations of BCAAs were shown in Fig. 1. As the concentrations of samples increased, the NO scavenging effect increased as well, showing concentration dependence. Figure 1 shows that the NO concentration of an SNP sample with a negative control was 137.20 μM, while an SNP sample mixed with 100 mM BCAAs produced approximately 100 μM of NO. There was no significant difference between the different BCAAs ($p$>0.05). However, it appeared that leucine had the greatest scavenging activity among BCAAs, followed by isoleucine and valine. That is, a reaction with 100 mM leucine produced only 98.52 μM of NO.

The amount of nitrite generated from the decomposition of SNP was found to be reduced significantly by BCAAs (22). Nakagawa and Yokozawa (23) reported that NO react rapidly with $O_2^-$ to form ONOO. $ONOO^-$ as a metabolite of NO and $O_2^-$ is highly reactive and directly induced toxic reactions. They also investigated that there are three proposals how tannin in green tea has NO scavenging effect: 1) the components of green tea tannin may removes NO and $NO_2^-$, which are precursors of $ONOO^-$, through a direct scavenging action; 2) tannin scavenges $ONOO^-$ itself; 3) tannin scavenges ·OH, which is known as a decomposition product of $ONOO^-$. As they mentioned how green tea tannin scavenges NO, there is possibility that BCAAs had scavenging effect over NO with those proposals as well. However, further studies are required to investigate exact mechanisms of NO scavenging.

A great number of naturally occurring substances have been recognized to have antioxidative abilities, and various

*in vitro* methods have been used to assess their free radical scavenging and antioxidative activities (24). Therefore, these results suggested that BCAAs scavenged the nitrite radicals, showing that they are potent antioxidants. Therefore, BCAAs can be used in inhibiting or slowing the progress of aging and age associated oxidative stress (25). They also have potential as natural antioxidants that could be used as additives to prevent food deterioration, replacing synthetic antioxidants, which are known to have many side effects (22). Further studies are required to clarify the anti-inflammatory effect and to improve the antioxidative activities of BCAAs for practical application in the functional food industry.

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