In Vitro Antioxidant Activities of Water-soluble Nucleotide-extract from Edible Fungi

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In this study, we obtained water-soluble nucleotide-extract from six kinds of edible fungi (Agrocybe chaxingu, Lentinus edodes, Coprinus comatus gray, Agaricus bisporus, Armillariella mellea, Flammulina velutipes), and the extract from Agrocybe Chaxingu sporocarp exhibited the highest total reducing power and the most remarkable scavenging activity on 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), which had a good dose-response relationship with the concentration of water-soluble nucleotide. When the concentration of the nucleotide was 20 mg/mL, the ABTS scavenging rate could be 90%. The nucleotide-extract was also found to exhibit remarkable scavenging activity on hydroxyl radicals (EC50 = 18.5 mg/mL), superoxide anion radicals (EC 50 = 38.4 mg/mL) and lipid peroxidation inhibition activity (EC50 = 8.1 mg/mL). Moreover, the main nucleotides in the nucleotide-extract were identified by HPLC, which were respectively AMP, CMP, GMP, UMP, ADP, GDP and GTP in a molar ratio of 1:2.17:3.49:1.76:0.75:4.18:1.67. These results indicated that the nucleotide would be a new antioxidant with wonderful prospects.

Keywords: Agrocybe Chaxingu, antioxidant activity, edible fungi, free radical, water-soluble nucleotide

Introduction

Free radicals are defined as any molecules or atoms having an unpaired electron in the outer orbit. With the possession of the unpaired electrons, free radicals are generally unstable and very reactive to cause the oxidation of biomolecules (e.g., protein, amino acids, lipid, and DNA), which leads to cell injury and death (Fang et al., 2002). Antioxidants can scavenge free radicals and inhibit tissue damage. In addition, natural antioxidants are safer and healthier than synthetic antioxidants which are used in food (Velioglu et al., 1998). The search for sources of natural antioxidants with radical scavenging activity in recent years has led to the discovery of some antioxidant substances such as phenolic compounds (Su et al., 2009; Bijak et al., 2011), Phellinus merrillii extracts (Chang et al., 2007), boiling water extracts of lotus seeds (Yen et al., 2005), ferulic acid (Prasad et al., 2007), and whey proteins (Tong et al., 2000).

The edible fungus is purely natural and pollution-free and it is always used for diet therapy and daily health care. Some edible fungi have currently been found to possess antioxidant activities (Oliveir et al., 2007; Lee et al., 2008). Mau et al. (2004) found that methanol extracts from mushroom mycelia showed high antioxidant activities. Similarly, Jia et al. (2007) concluded that methanol extract from Cassia tora L. by liquid-liquid partition using ethyl acetate exhibited more antioxidant potency than n-butanol and water fractions, and was more effective in protecting LDL against oxidation in a concentration-dependent manner. Moreover, the extract from fruit bodies of the Agrocybe contained the highest amount of total antioxidant components, and their antioxidant properties were more effective than filtrate of mycelia (Lo et al. 2005). However, different antioxidant compounds have been found in edible fungi such as the phenolic compounds (Cheung et al., 2004), flavonoids (Lee et al., 2008), and tocols (Lee et al., 2008). The nutrition concept of the edible fungus is not only a food substance that is safe and healthy, but also a potential health-promoting food. Therefore, it is our task to research on their antioxidant activities and find appropriate methods to extract their antioxidant components.

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al., 2003; Yang et al., 2002; Cheung et al., 2005), polysaccharides (Chen et al., 2008), triterpenes (Elmastas et al., 2007), ergosterol, and nicotinic acid (Hu et al., 2006; Zhang et al., 2003; Kobori et al., 2007) except nucleotide. Among all the nutrients, nucleotides, the basic units of genetic material, may be a conditionally essential nutrient. Increased need for exogenous nucleotides is likely to occur in certain circumstance, such as when the body is in rapid growth or suffers from an immunological challenge, intestinal injury or liver dysfunction. With further study for the past few years, its new functions have been discovered gradually. Exogenous nucleotides are important in maintaining optimal humoral immune responses (Jyonouchi, 1994). It seems to promote the rapid growth and physical maturation of bowel. (He et al., 1994). Also, a supplement of adenine nucleotides could help improve the resistance of liver cells in mice to toxins (Oyaizu, 1986). The distilled water and 1 mL of 0.1% ferric chloride. The supernatant mixture was then incubated for 10 min at room temperature. The absorbance (A) of supernatant mixture was measured at 700 nm with water used as the blank. The reducing ability varies directly with the absorbance of the reaction solution and a higher absorbance correlates with a more powerful reducing ability (Oyaizu, 1986). The distilled water was used as the negative control and the absorbance (A₀) was measured. The total reducing ability was described as A₁ − A₀. Ascorbic acid (VC) was used as the positive control.

**ABTS scavenging assay** ABTS scavenging assay was based on the slightly modified method of Zhao et al. (2010). ABTS radical stock solution was prepared with distilled water and stored at 4°C. According to the requirement, the ABTS solution was diluted to an absorbance of 0.70 ± 0.02 as working solution, just before use. Accurately transfer 0.1 mL of various concentrations of nucleotide-extract solutions (5, 10, 20, 30, 40 and 50 mg/mL) into a 10 mL tube
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with cap, respectively, and then add 1.9 mL ABTS working solution. After incubating for 6 min, the absorbance \((A_0)\) of the mixture was measured at 734 nm. The absorbance \((A_s)\) was measured by using distilled water replacing nucleotide-extract solutions. The absorbance \((A_t)\) was measured by using distilled water replacing ABTS solution to eliminate interference of the sample. ABTS scavenging activity of different concentrations of nucleotide extract was calculated by the following equation:

\[
\text{Rate} \%(\%) = \left(1 - \frac{(A_t - A_s)}{A_t}\right) \times 100\%
\]

VC was used as the positive control.

•OH scavenging assay  The •OH scavenging activity of the nucleotide was evaluated according to the reaction between sodium salicylate and residual •OH. For the determination of the •OH scavenging capacity of nucleotide, the sodium salicylate method was used, as described by Wang et al., (2008), but slightly modified. •OH was generated by Fenton reaction in the system of FeSO₄ and H₂O₂. The reaction mixture consisted of 1.0 mL FeSO₄ (6 mM), 1.0 mL H₂O₂ (6 mM) and 1.0 mL of various concentrations nucleotide-extract from Agrocybe chaxingu (NEA). The mixture stood for 10 min and then was added 1.0 mL sodium salicylate (6 mM). The total mixture stood again for 30 min and then the absorbance \((A_t)\) of the mixture was recorded at 510 nm with the distilled water as the blank. The absorbance \((A_s)\) was recorded using distilled water replacing 1.0 mL NEA and the absorbance \((A_0)\) was also recorded by using sodium salicylate replacing distilled water. VC was used as the positive control. The •OH scavenging activity was calculated using the following equation:

\[
\text{Rate} \%(\%) = \left(1 - \frac{(A_t - A_s)}{A_t}\right) \times 100\%
\]

O₂⁻ scavenging assay  O₂⁻ was generated by auto-oxidation of pyrogallol reaction (Liu et al., 1997). In brief, 0.2 mL of various concentrations NEA (10, 20, 30, 40 and 50 mg/mL) was mixed well with 5.7 mL of 50 mM Tris – HCl buffer (pH 8.2). The reaction was started by adding 0.1 mL of 6 mM pyrogallol to mixture and the reaction mixture was incubated at 25°C for 10 min. The absorbance \((A_t)\) of the reaction mixture was recorded at 320 nm every 30 s until the reaction proceeded to 6 min. The absorbance \((A_s)\) of different concentrations of NEA was recorded by using distilled water replacing the pyrogallol to eliminate the interference. The absorbance \((A_0)\) was measured by using distilled water replacing the NEA. The O₂⁻ scavenging activity was calculated by the following equation:

\[
\text{Rate} \%(\%) = \left(1 - \frac{(A_t - A_s)}{A_t}\right) \times 100\%
\]

VC was used as the positive control.

Lipid peroxidation inhibition assay  Lipid peroxidation inhibition assay was based on the slightly modified method of Li et al. (2009) and Lúcio et al. (2009). Egg lecithin (300 mg) was dispersed in a sodium phosphate buffer (30 mL, 10 mM, and pH 7.4) and oscillated in a water bath. TCA-TBA-HCl mixture was made by accurately weighing 15 g trichloroacetic acid (TCA), 0.37 g thiobarbituric acid (TBA), 2.1 mL HCl into a 100 mL volumetric flask and making up to the scale reading with distilled water. The mixture was dissolved enough by magnetic stirrers and stored at 4°C, before use. An adequate amount of egg lecithin solution was transferred into a petri plate and UV-radiated under 1.20 J m⁻²·s⁻¹ for 60 min through ultraviolet lamp and then left to stand in the darkness for 10 min.

Egg lecithin solution (1 mL) which was radiated was mixed with various concentrations NEA (0.1 − 24 mg/mL) and 2 mL TCA-TBA-HCl mixture. The mixture was maintained in 100°C water bath for 15 min, cooled quickly to room temperature and then centrifuged at 3000 rpm for 10 min. The absorbance \((A)\) of the supernatant was measured at 532 nm (the egg lecithin solution without radiation as blank). The absorbance \((A_0)\) was measured by using 1 mL PBS buffer replacing sample. Lipid peroxidation inhibition of different concentrations of nucleotide extract was calculated by the following equation:

\[
\text{Rate} \%(\%) = \left(1 - \frac{A}{A_0}\right) \times 100\%
\]

VC was used as the positive control.

HPLC determination of nucleotides  HPLC analysis was conducted on Agilent 1100 liquid chromatograph equipped with a Model G1311A quat solvent pump, a G1313A autosampler, a G1315B diode array detector. Agilent Eclipse XDB C18 reversed-phase packing columns (4.6 × 250 mm, 5 μm) were used for analysis. The column temperature was kept at 25°C. An injection loop of 20 μL was used. The DAD acquisition wavelength was 260 nm. A gradient elution was performed by varying the proportion of solvent A (10 mM ammonium acetate, pH 8.0) to solvent B (methanol), with a flow rate of 1 mL/min. The gradient elution program was set as follows: 15 min linear gradient from 5 − 15% B. All the prepared solutions were filtered through 0.45 μm membranes (Fisher Scientific) and the mobile phase was degassed before injection onto HPLC.

Retention times, identifications, and concentrations of individual components in nucleotide-extract from Agrocybe chaxingu was computed and recorded immediately by the recording integrator according to seven external nucleotide standards. 5′-monophosphate nucleotides of adenine (A), guanine (G), cytosine (C), and uracil (U), 5′-diphosphate nucleotide (ADP and GDP), and triphosphate nucleotide (GTP)
were purchased from Sigma-Aldrich Co., USA.

Results

Total nucleotide content Gridding beads were used for extraction and the extract yield was 6.94 ± 0.08 g/100 g. This solvent system with assisted grind destroys the cell membranes, simultaneously dissolving the nucleotide compounds. The nucleotide content of edible fungi extract was measured by the perchloric acid method and the total nucleotide content in each extract was 72.9 % (Agaricus bisporus), 70.8 % (Agrocybe chaxingi), 66.4 % (Armillariella mellea), 65.7 % (Coprinus comatus gray), 69.1 % (Flammalina velutipes), 63.6 % (Lentinus edodes), respectively. The result showed that the extract from edible fungi contained high nucleotide content.

Total reducing ability The reducing power of nucleotide-extract which was extracted from different edible fungi was presented in Fig. 1. Nucleotide-extract from different edible fungi was found to have different reducing ability and the reducing ability of nucleotide-extract from Agrocybe was the strongest. It was found the reducing ability was increased with the concentration of nucleotide-extract increasing. It was also shown that the nucleotide was the electron donor because the Fe3+ in potassium ferrocyanate was reduced to Fe2+ at low concentration of NEA. It was reported that the reducing ability of biochemical activities had a certain relationship with antioxidant ability (Meir et al., 1995; Juntachote and Berghofer, 2005). The reducing ability of nucleotides may enhance its antioxidant ability, though it was lower than VC. In this test, VC has reached a reducing power value of 0.24, 0.53 and 1.19, respectively at concentration of 0.01, 0.03 and 0.15 mg/mL.

ABTS scavenging activity The free radical scavenging activity of nucleotide-extract from different edible fungi was determined by the ABTS method and results were shown in Fig. 2. The ABTS is the most widely used and most stable chromogen compound to measure the antioxidant activity of biological material. High radical scavenging ratio indicates that the mechanism of antioxidant action of nucleotide was as a hydrogen donor and it could terminate the oxidation process by converting free radicals to the stable forms.

All nucleotide-extracts had obvious ABTS scavenging activity and the scavenging activities were almost the same in Agrocybe, Agaricus and Armillaria mellea sporophore. It was also shown that the higher the concentration of nucleotide was, the lower the amount of remaining ABTS was and the higher the free radical-scavenging activity was. The scavenging rate would be below 40% at the concentration of 0 – 5 mg/mL, while VC at concentration of 0.08 and 0.1 mg/mL, the positive control used in this test, had the scavenging rate of 78.7 and 99.8%, respectively. The ABTS scavenging rate would be increased obviously, when the concentration was higher than 5 mg/mL and it was 85.5% when the concentration was 10 mg/mL. For Agrocybe, Agaricus and Mel-lea armillaria sporophore, the ABTS scavenging rate could be 90% when the concentration was 20 mg/mL. The results showed the nucleotide-extract from the edible fungi was a nice ABTS scavenger.
•OH scavenging activity  •OH is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells. The hydroxyl radical •OH scavenging activity of NEA was shown in Fig. 3. NEA showed some hydroxyl radical scavenging activity but weaker than that of VC. When the concentration of nucleotide-extract was below 50 mg/mL, the higher the concentration was, the more powerful the free radical scavenging activity was. The rate increased obviously, when the concentration varied from 5 to 10 mg/mL, and then slowly when the concentration was higher than 30 mg/mL. The rate was 83.3% when the concentration was 30 mg/mL. All the results showed NEA had strong •OH scavenging activity, with an EC50 = 18.5 mg/mL.

O2ˉ scavenging activity  Oxygen is the most important electron acceptor in the organisms and it can form various lively oxygen radicals by accepting different number of electrons. Oxygen radicals include O2ˉ, organic peroxide radical and lipid peroxide radical and so on. Among the oxygen radicals, O2ˉ is the most harmful to organisms. It is the product of the one-electron reduction of O₂, which occurs widely with anoxia or ischemia in organisms. With one unpaired electron, the superoxide ion is a free radical and biologically quite toxic. Because O2ˉ is toxic, nearly all organisms living in the presence of oxygen contain isoforms of the superoxide scavenging enzyme, such as superoxide dismutase (SOD). Because of anoxia or ischemia, the amount of the adenosine triphosphate (ATP) is decreased in organisms. This caused the scavenging ability of the O₂ˉ scavenging enzyme to decrease and thus the oxyradical produced by organisms could not be scavenged. Then the organism is damaged and caused lipid peroxidation. Therefore, it is important for superoxide anion scavengers to protect the organisms, maintain cell membrane permeability, and maintain the organism's metabolic balance.

Pyrogallol is stable under acid conditions and will be automatically oxidized under weak alkaline conditions (Tris-HCl buffer, pH 8.2). During automatic oxidation, O2ˉ is produced continuously and the continuously produced O2ˉ is automatically oxidized further and produces some chromatic intermediate products. The intermediate products will be changed to other intermediate products through further oxidation (Agarwal et al., 2000). In our experimental system, we used pyrogallol automatic oxidation system to study the activity of superoxide anion radical scavenging of NEA. Record the absorbance at 320 nm every 30 s after different concentration of pyrogallol was added. O2ˉ scavenging activity was expressed by the oxidation degree, and the results were shown in Fig. 4. The absorbance increased gradually with the reaction time expanded, showing that the oxidation reaction proceeded. Moreover, it was observed that the absorbance decreased with the increase of the concentration of NEA, which meant the reduction of oxidation degree, indicating O2ˉ scavenging activity was stronger. The data at 5 min were adopted to evaluate the inhibition activity and the relationship between concentration and the inhibition activity was

Fig. 3. Dependence of the •OH scavenging activity of nucleotide-extract from Agrocybe Chaxingu sporocarp on the concentration. VC was used as the positive control. ○, nucleotide-extract from Agrocybe Chaxingu (NEA); ◆, VC.

Fig. 4. Different concentration of nucleotide-extract from Agrocybe Chaxingu sporocarp was respectively added to pyrogallol autoxidization system. Record the absorbance at 320 nm every 30 s after the autoxidation reaction started. The larger absorbance, the stronger oxidation. ○, Control; ●, 10 mg/mL; □, 20 mg/mL; ■, 30 mg/mL; △, 40 mg/mL;▲, 50 mg/mL.
nucleotide profiles of nucleotide-extract from *Agrocybe chaxingu*. The nucleotide components in the sample were identified, according to a chromatogram of the mixed nucleotide standards using the Agilent Eclipse XDB C18 HPLC system. In comparison to the retention time of the nucleotide standards, the main nucleotides in water-soluble nucleotide-extract from Agrocybe chaxingu are AMP, CMP, GMP, UMP, ADP, GDP and GTP in molar ratio of 1:2.17:3.49:1.76:0.75:4.18:1.67.

NEA was found to have inhibition activity for O$_2$\(^{-}\) and this scavenging activity rose gradually with the concentration increasing, though the O$_2$\(^{-}\) scavenging activity was weaker than that of VC. The concentration was 30–40 mg/mL when the inhibition ratio was 50% (that is EC$_{50}$ = 38.4 mg/mL).

**Lipid peroxidation inhibition activity**  
In the biomembrane the polyunsaturated fatty acid is easily attacked by oxyradical to produce toxic lipid peroxide radicals. This causes the changes of membrane permeability and energy metabolism which cause cell damage. In this experiment, the nucleotide-extract from edible fungus was confirmed to have strong inhibition activity for lipid peroxidation and the inhibition activity had some relationship with the concentration of nucleotide-extract. However, the inhibition activity is weaker than that of VC. Figure 6 showed that a larger the concentration correlated with a bigger function in restraining the lipid peroxidation, which was produced from egg lecithin treated by UV ray. The inhibition rate increased quickly form 18.0 ± 0.6% to 81.8 ± 1.0% when the concentrations of the nucleotide-extract varied from 1 mg/mL to 16 mg/mL. Moreover, 91.4 ± 0.8% of the peroxidation was inhibited by NEA at 24 mg/mL. The inhibition ratio was 50% (that is EC$_{50}$ = 8.1 mg/mL), when the concentration was 8.1 mg/mL. There was speculation that NEA may possess anti-irradiation effects, because the peroxidation of egg lecithin was caused by UV.

**Identification of nucleotides**  
Figure 7 contains the nucleotide profile of nucleotide-extract from *Agrocybe chaxingu* by HPLC using Agilent Eclipse XDB C18 reversed-phase packing column. 1, CMP; 2, UMP; 3, GTP; 4, GMP; 5, GDP; 6, ADP; 7, AMP.
Discussions
At present, there are numerous studies suggesting that oxidants might contribute to a wide spectrum of human maladies including cancer, cardiovascular disease and a host of neurodegenerative conditions (Balaban et al., 2005). More research of the natural antioxidants can help lead to prevention of a variety of diseases associated with oxidative damages. The precious studies have reported that the nucleic acid and its related material could be used as endogenous free radical scavenging agents. Nitrogen and oxygen atoms of the nucleotide bases can capture free radicals formed in linoleic acid oxidation, integrate with Cu and Fe that could accelerate oxidation, and therefore reduce the damage of cytomembrane and DNA caused by lipid peroxidation. (Ames et al., 1981).

In this study, we detected the in vitro effect of nucleotide-extract from edible fungi for the first time, including total reducing ability, free radical scavenging activity and lipid peroxidation inhibition.

Firstly, we evaluated the total reducing ability of nucleotide-extract from six edible fungi, Agrocybe chaxingu, Lentinus edodes, Coprinus comatus gray, Agaricus bisporus, Armillariella mellea, Flammulina velutipes. The total reducing ability of Agrocybe was the strongest among doses used in this work (from 5 to 40 mg/mL) compared with other edible fungi, though the ability of each nucleotide-extract was lower than that of VC. After that, we compared ABTS scavenging activity of nucleotide-extract from six edible fungi. The obtained result clearly indicated the scavenging properties of three used edible fungi nucleotide-extract, Agrocybe chaxingu, Agaricus bisporus, and Armillariella mellea. Further study was to verify this antioxidant action on Agrocybe chaxingu due to the strongest total reducing ability and better ABTS scavenging activity, including \( \cdot \)OH scavenging, \( \cdot \)O\(_2\)\(^{-}\), scavenging, and lipid peroxidation inhibition activities. Our results confirmed that nucleotide-extract possessed an activity of antioxidants and the higher content of nucleotide compounds in the extract might account for their improved antioxidant activity.

Taken together, the antioxidant results indicated that nucleotides-extract from edible fungi demonstrated the antioxidant effect. However, nucleotides showed only a mild antioxidant activity, comparable to VC, a common antioxidant. The antioxidant activity of nucleotide-extract from Agrocybe chaxingu suggested that the consumption of Agrocybe chaxingu as daily food or an ingredient in food preparation may contribute a potential health benefit as an antioxidant, promoting cancer prevention.

In the present report, the content of total nucleotide in each crude extract from edible fungi was high (around 70 %). Both the type and ratio of nucleotides in the extract from Agrocybe chaxingu had been identified by HPLC. However, it was any one nucleotide or mixed nucleotides in this ratio that provided the antioxidant ability. Correlation studies are in progress. In any case, the present study on edible fungi can provide an experimental basis about the virtues of nucleotide-extract, used in folk food or medicine as a natural antioxidant.

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