3-Hydroxy-6-Methylpyridine with Preventive Activity on Carbon Tetrachloride-Induced Liver Injury Is Produced During Roasting of Coffee Beans

Kiharu Igarashi1*, Chihiro Kawai1 and Shizue Kurakane2

1 Department of Bioresource Engineering, Faculty of Agriculture, Yamagata University, 1-23 Wakaba-machi, Tsuruoka-shi, Yamagata 997-8555, Japan
2 Course of the Science of Bioresource, The United Graduate School of Agricultural Science, Iwate University, Morioka, Iwate 020-8550, Japan

Received January 10, 2010; Accepted September 24, 2010

Compounds which can be formed over the course of roasting coffee beans were investigated and isolated. One of these compounds was identified as 3-hydroxy-6-methylpyridine (3,6-Py). 3,6-Py and the coffee prepared from light-roasted coffee beans which contain this compound tended to suppress increases in plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities induced by carbon tetrachloride in mice, indicating that 3,6-Py formed during the roasting of coffee beans may be able to mitigate liver injury. The amounts of 3,6-Py were higher in French-roasted than light-roasted coffee beans of the Brazil Santos and Colombia Excelso varieties, suggesting that this compound might be produced under a more severe roasting condition. However, suppressive activity against liver injury was stronger in coffee from light-roasted than French-roasted beans. As the amounts of chlorogenic acid, which is known to have hepatoprotective activity, were higher in coffee from the light-roasted than French-roasted beans, stronger hepatoprotective activity in the coffee from light-roasted beans might be mainly concerned with its higher content of chlorogenic acid.

Keywords: roasted coffee beans, 3-hydroxy-6-methylpyridine, liver injury, roasting of coffee beans

Abbreviations: FAB-MS, fast atom bombardment mass spectrometry; DPPH, 1,1-diphenyl-2-pycrylhydrazyl; TE, trolox equivalency.

Introduction

In ancient times, coffee was drunk for medicinal purposes. Recently, however, coffee has become commonplace in Japan so much so that Japan has become the fourth largest coffee consumer in the world. As a result of its newfound popularity, many researchers have studied coffee components and their physiological functions. In particular, many physiological and pharmaceutical functions of caffeine, a major component in coffee beans, were studied in relation to the benefits of coffee consumption. Caffeine, although suspected to be a cause of cancer, has been shown to have no effect on cancer. On the other hand, coffee has been found to suppress the occurrence of stomach and liver cancers (Bauer et al., 1977; Mohr et al., 1984; Inoue et al., 2005). Instant coffee, and coffee diterpenes, kahweol and cafestrol, and chlorogenic acid are reported to prevent acute liver injury induced by carbon tetrachloride (CCL4) (Ozercan et al., 2006; Lee et al., 2007; Kapil et al., 1995; Zhou et al., 1993). Moreover, coffee has been reported to be effective in the prevention of liver injury and the mitigation of type 2 diabetes (Dam and Feskens, 2002), although the validity of these findings is still under debate (Ruhl and Everhart, 2005; Haner et al., 2008). Reports pointed out that it is necessary to determine the principles by which these effects are demonstrated and to verify such effects using such principles. There are many unidentified compounds in coffee beans. From roasted beans, we previously isolated and identified one of these compound as 3-pyridinol (3-Py) (Kurakane and Igarashi, 2006), which was found to be effective in the prevention of oxidative stress. Furthermore, its related compounds are also produced during the process of roasting coffee beans (Kurakane and Igarashi,
2006).

In this report, we identified and isolated another compound in the roasted beans and subsequently determined its contents and preventive effect against CCl4-induced liver damage in mice.

Materials and Methods

Isolation of 3-hydroxy-6-methylpyridine and its related compounds from roasted coffee beans  Light- and French-roasted coffee beans were prepared by roasting decaffeinated beans of the Colombia Excelso variety at 200°C for 14 min, and 220°C for 17 min, respectively. To isolate the coffee constituents of the roasted coffee beans, chilled hot water extracts from decaffeinated and light-roasted coffee beans were placed in a Diaion column (20 i.d. × 200 mm, Mitsubishi Chemical Co., Tokyo, Japan) and then eluted with 50% MeOH after washing the column with distilled water. The eluate was evaporated to remove MeOH in a vacuo and then extracted with diethyl ether. The diethyl ether layer was evaporated after being dried with Na2SO4 and subsequently dissolved in a small amount of MeOH for a preparative TLC with silica gel as an adsorbent and ethyl acetate:CHCl3:88% HCOOH:H2O (19:1:1:1, v/v) as a developing solvent. Each region corresponding to the compounds (compounds a and b) and exhibiting orange and pink colors on the TLC plate after a visualization of diazotized sulphanilic acid spraying reagent (each, Rf: 0.32 and 0.15) were scraped from the TLC plate. Each compound was eluted from the scraped silica gel with a mixture of MeOH and ethyl acetate (1:1, v/v) and then purified further using Sephadex LH-20 (15 i.d. × 900 mm, GE Healthcare BioSciences AB, Japan) column chromatography with MeOH as the developing solvent. Each fraction containing compounds a and b was further purified by a preparative HPLC with a Develosil C-30-UG-5 column (20 i.d. × 250 mm). Elution from the column was performed using 5% MeOH in water from 0 to 75 min, 50% MeOH in water from 75 to 230 min, and 100% MeOH after 230 min. The fractions containing compounds a and b were combined and then evaporated. The chemical structures of the obtained compounds were determined by NMR and MS spectra analyses. The NMR spectra were recorded on a JNM-EX 400 FT-NMR spectrometer (JEOL, Tokyo, Japan) using CD3OD as the solvent. The FAB-MS spectrum was recorded on a JMX-D 300 spectrometer (JEOL, Tokyo, Japan) with glycerol as the matrix.

Measurement of 3-hydroxy-6-methylpyridine (3,6-Py), 3-pyrnidinol (3-Py), and chlorogenic acid contents in coffee beans  For the measurement of 3,6-Py and 3-Py contents in coffee beans, decaffeinated light- and French-roasted Colombia Excelso coffee beans along with Brazil Santos coffee beans (without decaffeination) were used. Each bean (10 g) was extracted with 150 ml hot water using a coffee maker. The filtrate of the extracts (drip coffee) was subjected to HPLC using a Develosil ANIDIEU column (4.6 i.d. × 250 mm, Nomura Chemical Co., Aichi, Japan) for the measurements of 3,6-Py and 3-Py. Elution of each compound from the column was performed using 95% acetonitrile in water, and detection occurred at 290 nm. Commercial 3,6-Py and 3-Py were used as the standard for the calculation of each compound after dissolution in water.

Chlorogenic acid in the filtrate was measured using a Develosil C-30-UG-5 column (4.6 i.d. × 250 mm) and a solvent system composed of 5% acetonitrile in 1% acetic acid (solvent A) and 40% acetonitrile in water (solvent B). A linear gradient of 0-100% solvent B in solvent A over the course of 180 min at a flow rate of 0.8 mL/min was used. Chlorogenic acid was detected at 330 nm.

Inducement of liver damage by CCl4  Preventive effects of compounds a and b, in addition to light- and French-roasted coffee, on liver injury were determined as follows. Eight-week old male ddY mice (Japan SLC) were acclimated for 5 days in an environment with a temperature of 22 ± 2°C, 40-60% humidity and light cycle of 6:00-18:00. After feeding on a basal diet for 3 days, the mice were divided into six groups: the control group (5 mice), the liver injury group by CCl4 (CCl4 group, 8 mice), the CCl4+3-Py group (3-Py group, 7 mice), the CCl4+3,6-Py group (3,6-Py group, 8 mice), the CCl4+light coffee (freeze-dried coffee prepared from drip coffee from decaffeinated and light-roasted Colombia Excelso beans) group (DCL group, 7 mice) and CCl4+French coffee (freeze-dried coffee prepared from drip coffee from decaffeinated and French-roasted Colombia Excelso coffee beans) group (DCF group, 7 mice). After fasting for 8 h, mice were administered one of the following solutions: to the control and CCl4 groups, 0.2 mL of 0.5% sodium carboxymethyl cellulose (CMC) solution; to the 3-Py group, 0.2 mL 3-Py suspension prepared by suspending 100 mg 3-Py in 1 mL of 0.5% CMC solution; to the 3,6-Py group, 0.2 mL 3,6-Py suspension prepared by suspending 100 mg 3,6-Py in 1 mL of 0.5% CMC solution (in equal moles with 3-Py); and to the DCL and DCF groups, 0.2 mL of 0.5% CMC solution containing 100 mg/ml freeze-dried coffee prepared from decaffeinated and light- or French-roasted Colombia Excelso beans as described above. The amounts of 3-Py and 3,6-Py contained in the administered solution were 0.08 and 0.01 mg in the light-roasted beans, and 0.07 and 0.02 mg in the French-roasted beans, respectively.

The control group was injected intraperitoneally with 30 µL olive oil 30 min after the administration of 0.5% CMC solution. The other groups were injected with a mixture of 30
spectra data also confirmed the chemical structure of compound b to be 3,6-Py. This is the first report to identify this compound in raw and roasted coffee beans.

**Chemical structure of the compounds related to 3-Py**

Since the Rf value of compound a (0.32) and its color upon being sprayed with diazotized sulphanilic acid on the TLC plate was the same as those of authentic 3-Py, which was previously isolated from roasted coffee beans (Kurakane and Igarashi, 2006), Compound a was identified as 3-Py.

Compound b (Rf 0.15) exhibited a pink color when sprayed with diazotized sulphanilic acid, suggesting the presence of more than a phenolic hydroxyl group in its chemical structure. The FAB-MS spectrum of compound b showed a pseudo-molecular ion peak at m/z 110 (M+H)+, indicating a molecular weight of 109. The 13C-NMR spectrum of compound b measured in CD3OD showed the same spectrum with that of authentic 3-hydroxy-6-methylpyridine (3,6-Py) [100 MHz, δc (CD3OD): 22.5 (CH3), 125.3 (C-4 or C-5), 125.4 (C-5 or C-4), 136.9 (C-2), 149.5 (C-6), 153.6 (C-3)]. The 1H-, 1H-1H COSY, 1H-13C COSY, and HMBC NMR

![Chemical structures](image)

**Fig. 1.** Chemical structures of 3-pyridinol (3-Py) and 3-hydroxy-6-methylpyridine (3,6-Py).
observed in all of the coffee samples except those prepared from raw beans. The relationship between the contents of these compounds and roasting conditions are shown in Table 1. Although the amounts of 3-Py in Colombia Excelso was almost the same between the light- and French-roasted beans, the amounts of 3,6-Py was higher in the French-roasted beans. As the amounts of 3,6-Py in Brazil Santos was also higher in the French- than light-roasted, 3,6-Py was thought to be produced when beans were roasted under severe roasting conditions. The higher amounts of 3-Py, compared to those of 3,6-Py, in both the light- and French-roasted coffee, suggest that this compound has been produced in a relatively mild roasting condition compared to the conditions for producing 3,6-Py.

The formation pathway of 3,6-Py is very interesting because of its similar chemical structure with 3-Py which was previously isolated from roasted coffee beans (Kurakane and Igarashi, 2006). The methylation of 3-Py on its 6-position under severe roasting conditions may be a cause of the formation of 3,6-Py because of the fact that its content increased in the French-roasted coffee. However, the precise formation mechanism still remains to be clarified.

The effects of 3,6-Py, and coffee prepared from beans with different roasting conditions on CCl4-induced liver injury The plasma ALT and AST activities of mice administered each coffee sample are shown in Fig. 3. Both 3,6-Py and coffee from light-roasted coffee beans suppressed an increase in the AST activities induced by CCl4. ALT activity tended to be suppressed by administrating 3,6-Py (0.05 < p < 0.1). Suppression by 3-Py was much weaker than that of 3,6-Py. Although 3,6-Py showed a suppressive effect for increasing the ALT and AST activities caused by CCl4, the content in 20 mg freeze-dried coffee extract which was given to each mouse was 0.01-0.02 mg. This amount is considered to be too small to demonstrate the preventive effects against liver injury. Therefore, the other compounds such as chlorogenic acid, kahweol and cafestrol contained in coffee beans, which are known to be effective in mitigating CCl4-induced hepatotoxicity (Shi et al., 2009; Lee et al., 2007), might have contributed more strongly to the preventive effects of coffee extracts. Stronger suppressive effects found in light-roasted beans, compared to those in French-roasted beans with higher 3,6-Py content, may support the idea that coffee contains other compounds useful for the prevention of liver injury. Chlorogenic acid content in coffee, prepared from 100 g light- and French-roasted beans was 6.6 and 1.3 g, respectively. These amounts were much higher than those of 3,6-Py in the same coffee samples. Therefore, the stronger suppressive effects of coffee against liver injury from the light-roasted beans, compared with that from the French-roasted beans, might be mainly due to the higher content of chlorogenic acid in the light-roasted coffee, as chlorogenic acid is known to be available for the prevention of liver injury (Kapil et al., 1995; Zhou et al., 1993).

The DPPH radical scavenging activity of chlorogenic acid (1.14 µmol TE/µmol sample) was stronger than that of 3-Py (9.09 × 10-3 µmol TE/µmol sample) when measured using the DPPH method (Takahata et al., 2001). This result may also support that the stronger suppressive effects of coffee from the light-roasted beans, compared with that from the French-roasted beans, is mainly due to the higher content of chlorogenic acid in coffee from the light-roasted beans, although the suppression of liver injury by the other constituents cannot be excluded. However, the preventive effects of 3,6-Py, contained only in small amounts, may be important from the standpoint that the roasting process produced hepatoprotective compounds.

In this paper, it was reported for the first time that 3,6-

<table>
<thead>
<tr>
<th>Table 1. Relation between the roasting conditions and contents of 3-hydroxy-6-methylpyridine and 3-pyridinol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colombia Excelso</td>
</tr>
<tr>
<td>Raw beans</td>
</tr>
<tr>
<td>3-hydroxy-6-methylpyridine (mg/100 g beans)</td>
</tr>
<tr>
<td>3-pyridinol (mg/100 g beans)</td>
</tr>
</tbody>
</table>

Light-roasted and French-roasted beans were prepared by heating beans at 200°C for 14 min, and at 220°C for 17 min, respectively. Coffee was prepared by extracting 10 g decaffeinated roasted beans (Colombia Excelso), roasted beans (Brazil Santos), or raw beans (Colombia Excelso) with 150 mL boiling water using a coffee maker. nd: not detected. Values for decaffeinated Colombia Excelso and Brazil Santos are means of 3 and 5 measurements, respectively. Data are means ± S.E.
Py, which was produced in the course of roasting of coffee beans, was effective for mitigating liver injury, although its content in the roasted beans is very low.

Acknowledgement This study was supported by a grant from the All Japan Coffee Association.

References


