Detoxification of Ochratoxin A on Heating under Acidic and Alkaline Conditions

Alka B. Trivedi, Etsushiro Doi, and Naofumi Kitabatake*

Research Institute for Food Science, Kyoto University, Uji, Kyoto 611, Japan
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Ochratoxin A was heated under three different moisture conditions, and under acidic and alkaline conditions. Heating to 175°C under the dry conditions produced little change in the molecule and cytotoxicity, detected by TLC and in the effects on the proliferation of HeLa cells. When heated under the moist and watery conditions, a small change in molecule was found by TLC, but cytotoxicity was not reduced. Under the acidic conditions (0.1 N HCl) the decomposition of ochratoxin A was detected by TLC, however the change in cytotoxicity was not observed in this assay system. On the other hand, heating with NaOH (0.1 N) resulted in the decomposition and detoxification of ochratoxin A. The HPTLC analysis showed the formation of some decomposed compounds including L-phenylalanine. This indicates the hydrolysis of ochratoxin A is one of the decomposition reactions induced by alkali at high temperatures.

Ochratoxin A, a secondary metabolite of the fungi Aspergillus and Penicillium,1-3 is one of the seven mycotoxins that have been reported to occur significantly in naturally contaminated food and feed.4 Contamination of meat and meat products by ochratoxin A has been reported,5 indicating that feed might be highly contaminated by ochratoxin A. Ochratoxin A is 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3R methyl isoxoumarin linked to L-β-phenylalanine through the carboxyl group.6

Toxicological studies showed that the toxicity of ochratoxin A was associated with the phenylalanine moiety and chlorine group. The hydrolysis of ochratoxin A either by acid7 or carboxypeptidase A80 results in L-phenylalanine and the non-toxic isocoumarin acid (ochratoxin A).

Heat treatment has been proved effective in the elimination of antinutritional or toxic compounds in various foods.9 It is also effective for the detoxification of some of the mycotoxins. Heating at high temperatures for a long period of time was found to be effective in the elimination of aflatoxin and fusarium toxins.10,11 Heating above 140°C under semi-moist conditions detoxified citrinin.12 Ochratoxin A is a comparatively stable compound; even autoclaving for up to 3 hr was not found to be enough to destroy it completely, and in the presence of water it becomes much more stable.60 Hence, in this study, the possibility of detoxification of ochratoxin A by heating in the presence of water, weak acid, or weak alkali at a temperature higher than 100°C was investigated. The change in ochratoxin A content was observed by thin-layer chromatography (TLC) and high performance thin-layer chromatography (HPTLC) and the change in toxicity was measured by a bioassay using HeLa cells.

Materials and Methods

Materials. Benzene-free ochratoxin A was purchased from Makor Chemicals Ltd. (Jerusalem, Israel). Chloroform and ethanol of spectrophotometric grade were from Nacalai Tesque, Inc. (Kyoto). Dulbecco’s modified Eagles medium (type Auto Mod) of cell culture reagent grade and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemicals Co. (St. Louis, MO, U.S.A.), trypsin, isopropanol, and fetal bovine serum (FBS) were obtained from Gibco/BRL Life Technologies Inc. (Gaithersburg, MD, U.S.A.), Wako Pure Chemical Industries, Ltd. (Osaka), and Whittaker Bioproducts, Inc. (Walkersville, MD, U.S.A.), respectively. Other reagents were purchased from Nacalai Tesque and Wako Pure Chemical Industries. The HeLa cell line was obtained from Dainippon Pharmaceutical Co. Ltd. (Osaka).

Heating of ochratoxin A. The stock solution of ochratoxin A was prepared in chloroform (0.1 µg/µl) and stored at -20°C until use. The concentration of ochratoxin A was measured by UV absorption at 333 nm (ε 1% at 333 nm; 4,680 ± 40, in absolute ethanol13 using a spectrophotometer (UV-160A, Shimadzu Corp., Kyoto). One milliliter of stock solution was pipetted into a 1-mL glass vial (v-1, Nichiden Rika Glass Co., Ltd., Tokyo) and dried in a stream of dry N2. Then, 100 µl, 4 µl, or 0 µl of distilled water was added to the dried ochratoxin A in the vial and capped with an aluminum cap having silicon packing. These samples under three different moisture conditions; dry, moist, and watery, were heated as follows. The vials without water (dry condition) were placed in an oil bath and heated to 200°C at a rate of 3°C/min. The vials with 4 and 100 µl water (moist and watery) were heated in a thermo-regulated pressure vessel (type TEM-V, Taitatsu Scientific Glass Co., Ltd., Tokyo). The vials containing samples were placed in a vessel and heated to 175°C at a rate of 3°C/min. After the temperature of 175°C was reached, heating was stopped and cooling began. During the heating process, the temperature inside the vial was checked using a thermometer (Nichiyu, Tokyo).

After being cooled to room temperature, the samples were lyophilized.

When ochratoxin A was heated under acidic or alkaline conditions, 100 µl of 0.1 N HCl or 0.1 N NaOH was used instead of water. Other conditions including temperature were the same as those described above.

Analytical measurement of heated ochratoxin A. Analytical measurements were done by TLC, HPTLC, and UV spectrophotometry. For TLC, the lyophilized samples were solubilized with chloroform and 2 µg of the sample were spotted on thin-layer silica gel plates (E. Merck, P. O. B., Darmstadt, Germany) along with the ochratoxin A standard. The plate was developed in a solvent of ethyl acetate-toluene-formic acid (6:3:1). After drying, fluorescent spots were made visible with UV light (excitation 365 nm). For HPTLC, sample was solubilized in ethanol and spotted on an HPTLC aluminum sheet (silica gel 60 without fluorescent indicator, E. Merck). The plate was developed in a solvent of n-butanol-acetic acid-water (4:1:1), and the spots were observed in the same way as above. UV absorption spectra were obtained on a Shimadzu UV-VIS spectrophotometer UV-160A in absolute ethanol.

Cytotoxicity of ochratoxin A. The cytotoxicity of ochratoxin A and ochratoxin A heated with water, acid, or alkali was bioassayed using HeLa cells. The preparation and counting of HeLa cells were done as described before.14 Samples were solubilized in fetal bovine serum (FBS) and diluted

* To whom correspondence should be addressed.
with Dulbecco's modified Eagle's medium to make different concentrations. Then, 50 μl of the solution was put into a well in a microwell plate (96 wells, Nunc). To each well, 50 μl of cell suspension of 6 x 10^6 cells/ml was added. Cells in the microwell plate were incubated at 37°C and 5% CO₂ atmosphere. After 72 hr of incubation, the cell growth was measured by a colorimetric method with the use of MTT, which gave a good correlation between the cell number and color development upon the reduction of MTT under suitable conditions. The color intensity was measured by a microplate reader (type MPRA-4, Tosoh, Tokyo) at the test wavelength of 540 nm and reference wavelength of 620 nm.

All manipulations were done in subdued light or red light to prevent the photolysis of ochratoxin A.^

**Results**

Figure 1 shows the TLC chromatogram of ochratoxin A heated under various moisture conditions. Ochratoxin A heated under the dry condition to 200°C for 10 min showed a spot with an Rf value and fluorescence similar to those of the ochratoxin A standard. The samples heated under the moist or watery conditions gave similar spots to those of the ochratoxin A standard and other weak fluorescent spots with lower Rf values appeared. This indicates that the decomposition of ochratoxin A was little and was not enough to detoxify it.

As the heating of ochratoxin A under various moisture conditions even to the extreme temperature (200°C) could not cause any significant change or decomposition, heating under other conditions was done. When heated with 0.1 N HCl, ochratoxin A showed a spot similar to that of the ochratoxin A standard and another spot with a slightly higher Rf value was observed. When ochratoxin A was heated with 0.1 N NaOH to either 100°C or 175°C, it changed to another compound having a much lower Rf value than that of the ochratoxin A standard (Fig. 2).

The ochratoxin A heated with NaOH was developed with another solvent using an HPTLC silica gel sheet. Some fluorescent spots other than ochratoxin A appeared, indicating that there were several decomposed compounds (Fig. 3). Ochratoxin A produced a greenish-blue spot and other spots with lower Rf values than that of ochratoxin A had blue fluorescence. Since ochratoxin A is hydrolyzed by carboxypeptidase A, yielding L-phenylalanine and

**Fig. 1.** Thin Layer Chromatogram of Ochratoxin A and Ochratoxin A Heated under Various Moisture Conditions. A, ochratoxin A standard; B, ochratoxin A heated under the dry condition to 200°C for 10 min; C, ochratoxin A heated under the moist condition (100 μg ochratoxin A/4 μl water) to 175°C; D, ochratoxin A heated under the watery condition (100 μg ochratoxin A/75 μl water) to 175°C.

**Fig. 2.** Thin Layer Chromatogram of Ochratoxin A and the Ochratoxin A Heated at Various pHs under the Watery Condition. A, ochratoxin A standard; B, ochratoxin A heated with 0.1 N NaOH to 100°C; C, ochratoxin A heated with 0.1 N NaOH to 175°C; D, ochratoxin A heated with 0.1 N HCl to 100°C; E, ochratoxin A heated with 0.1 N HCl to 175°C; F, ochratoxin A heated with water to 100°C; G, ochratoxin A heated with water to 175°C.

**Fig. 3.** HPTLC of Ochratoxin A. Ochratoxin A Heated with 0.1 N NaOH, and L-phenylalanine. L-Phenylalanine (1), ochratoxin A heated with 0.1 N NaOH (2), and ochratoxin A standard (3) were spotted, and after development the TLC plate was seen by UV light (A) and was stained with ninhydrin (B).

**Fig. 4.** Ultraviolet Spectra of Unheated Ochratoxin A and Ochratoxin A Heated under Various Moisture Conditions. Ochratoxin A standard (——), ochratoxin A heated to 200°C for 10 min under the dry condition (-----), ochratoxin A heated under the moist condition (———), ochratoxin A heated under the watery condition (———).
Ochratoxin A, it is supposed that the same reaction could occur by heating with dilute NaOH. Therefore, the HPTLC sheet was stained with ninhydrin. The heated ochratoxin A gave a faint pink spot with ninhydrin staining and the same Rf value as that of L-phenylalanine (Fig. 3). Heating with NaOH definitely hydrolyzed ochratoxin A similarly to the carboxypeptidase A reaction. However, several fluorescent spots, other than the faint spot of L-phenylalanine, were observed (see Figs. 2 and 3). Therefore, the decomposition of ochratoxin A seems to have been caused not only by hydrolysis, but also by other unknown reactions.

Figures 4, 5, and 6 show the UV absorption spectra of ochratoxin A and ochratoxin A heated with water, NaOH, or HCl, respectively. Two absorption peaks, approximately at 219 and 333 nm were observed. Ochratoxin A heated with water also showed a UV spectrum similar to that of non-heated ochratoxin A. Only the ochratoxin A heated under the watery condition showed higher absorption, near 380 nm. Under alkaline and acidic conditions, the spectrum of ochratoxin A changed; absorption at a wavelength higher than 333 nm appeared. Although the ochratoxin A heated with 0.1 N NaOH gave a spot on TLC different from that of ochratoxin A, it yielded a similar absorption spectrum, showing the absorption peak at around 333 nm. When ochratoxin A was heated with 0.1 N HCl, the absorbance at 333 nm gradually decreased with an increase in heating from room temperature to 100°C and 175°C. Accompanying the decrease in absorbance at 333 nm, the absorbance at higher wavelengths gradually increased.

The cytotoxicity of ochratoxin A was examined on the proliferation of HeLa cells. Figure 7 shows the effects of different amount of ochratoxin A on cell growth with incubation up to 80 hr. Cells without ochratoxin A proliferated with time, which is evident from the gradual increase in the absorbance. Here, the absorption is related to the number of cells. Under these experimental conditions, 0.1 μg/well ochratoxin A did not show any toxic effect. That is, at 50 hr of incubation, the proliferation of the cells treated

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**Table 1.** Cytotoxicity of Ochratoxin A Heated under Acidic and Alkaline Conditions

<table>
<thead>
<tr>
<th>Concentration of ochratoxin A (μg/well)</th>
<th>H₂O</th>
<th>HCl</th>
<th>NaOH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100°C</td>
<td>175°C</td>
<td>100°C</td>
</tr>
<tr>
<td>0.0</td>
<td>0.245 ± 0.008</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>0.1</td>
<td>0.221 ± 0.027</td>
<td>0.231 ± 0.004</td>
<td>0.262 ± 0.007</td>
</tr>
<tr>
<td>0.5</td>
<td>0.152 ± 0.011</td>
<td>0.123 ± 0.004</td>
<td>0.167 ± 0.004</td>
</tr>
<tr>
<td>1.0</td>
<td>0.050 ± 0.011</td>
<td>0.055 ± 0.004</td>
<td>0.063 ± 0.000</td>
</tr>
<tr>
<td>3.0</td>
<td>0.051 ± 0.005</td>
<td>0.065 ± 0.006</td>
<td>0.071 ± 0.005</td>
</tr>
</tbody>
</table>

* Cytotoxicity was measured by the MTT method. The values given in the table are absorbance at 540 nm of reduced MTT, which corresponds to the number of cells. HeLa cells used were incubated with ochratoxin A for 72 hr. Triplicate experiments were done and the average and S. D. were shown. Details were given in the text.

*b* The ochratoxin A heated with 0.1 N HCl or 0.1 N NaOH was neutralized with NaOH or HCl before addition to cells, respectively.
with 0.1 µg/well ochratoxin A were slightly suppressed, but at the end of the incubation period, the absorbance of the well was the same as that of the control well to which ochratoxin A was not added. Addition of 0.5 µg/well suppressed the growth of the cells. The concentrations that were more than 0.5 µg/well had lethal effects. At 1 µg/well concentration, the absorbance of the well was the same as that of the blank well (i.e., without cells) at 50 hr of incubation.

The cytotoxicity of ochratoxin A heated under acidic, alkaline, or neutral conditions was examined (Table I). Heating at neutral (with water) or under acidic (0.1 N HCl) conditions, showed effects similar to that of the ochratoxin A standard. In other words, upon the addition of 0.5 µg/well, i.e., 5 ppm, ochratoxin A suppressed the growth of cells, and upon the addition of 1 µg/well, i.e., 10 ppm, ochratoxin A or of higher concentrations, had lethal effects. Ochratoxin A heated under alkaline conditions to 100°C and 175°C, both showed less toxicity than the ochratoxin A standard. Up to 1 µg/well concentration, no toxicity was found. However, at higher concentrations (3 µg/well), the lethal effect on the cells still remained.

**Discussion**

Treatment of food material with any chemical degrades quality and most of the time, the food does not remain suitable for human consumption. If such toxins could be eliminated by conventional cooking; boiling, frying, baking etc., or food processing (i.e., retort cooking, extrusion cooking etc.), it may be the best way. Citrinin can be decomposed to non-toxic compounds by heating to 140°C under moist conditions and this heating temperature and moisture can be used for ordinary cooking. Ochratoxin A seems to be more stable than citrinin or aflatoxin. Therefore, only heating may not be enough as in neutral condition where heating to extreme temperatures (200°C, 10 min) does not change/detoxify it. The effects of heating under acidic conditions is in good agreement with the study by Merwe et al. reporting that the degradation occurs, but it is very slow and not enough for detoxification. On the other hand, heating at 100°C with NaOH (0.1 N) yields the detoxifying effect to a large extent, although an addition of 0.1 N NaOH at room temperature does not give any detoxifying effect. When cells were treated with the same amount of NaOH, no toxic effect was produced. In this study only dilute HCl and NaOH were examined. The effects of acid and alkali must depend on the concentration, and acids and alkalis other than HCl and NaOH may also have other effects besides the effect of pH.

Trenk et al. reported that ochratoxin is more stable in the presence of water. However, our study shows that heating under dry conditions up to 200°C for 10 min does not make any change in ochratoxin, although in the presence of water, some other decomposed compounds were observed by TLC on heating to 175°C. The reason is that with an increase in water content, the decomposed compound also increased.

The cytotoxicity of another mycotoxin, citrinin, has been examined using HeLa cells with a method similar to the one described by Trivedi et al. In the case of citrinin, the addition of 2 µg/well (i.e., 20 ppm) suppressed the growth of cells, and with 5 µg/well (i.e., 50 ppm) lethal effects were observed. The LD₅₀ of citrinin for HeLa cells (unpublished data) is shown in Table II. According to the toxicity test using mice and rats, the LD₅₀ of citrinin was about 35 mg/kg-body weight (i.e., 35 ppm, and 67 mg/kg weight (i.e., 67 ppm) respectively, as presented in Table II. It indicates that the toxicity assay system using HeLa cells is more sensitive to these mycotoxins than the assay system using experimental animals, and that ochratoxin is more toxic than citrinin. The LD₅₀ of ochratoxin for mice, reported by Lindenfelser et al. who used the mixture of ochratoxin A (86%) and ochratoxin B (14%), is 24 ppm, which seems to be higher than that of ochratoxin A alone. The assay system using HeLa cells gives similar effects and tendencies as those results using experimental animals. That is to say, the toxicity of each mycotoxin for an animal corresponded well to the results obtained from the cell-culture system using HeLa cells.

The toxicity test using cultured cells is simple, reliable, and convenient for examining the toxicity of many samples. This method can be used for toxicological screening and is valuable for predicting the toxicological effects of tested samples. Although it is still important to study the validity of such a system using different kinds of cultured cells, the study of detoxification and decomposition of toxic compounds in food or feed by various methods should be further developed after the cell-culture system is better introduced in this field.

**Table II.** Toxicity (LD₅₀) of Ochratoxin and Citrinin

<table>
<thead>
<tr>
<th>Cells</th>
<th>Ochratoxin</th>
<th>Citrinin</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>5 ppm</td>
<td>19 ppm</td>
</tr>
<tr>
<td>Rat</td>
<td>20 ppm</td>
<td>67 ppm</td>
</tr>
<tr>
<td>Mouse</td>
<td>24 ppm</td>
<td>35 ppm</td>
</tr>
</tbody>
</table>

* Purches et al.\(^1\)
* Ambrose and DeEds\(^2\)
* Lindenfelser et al.\(^3\)

**References**

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