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# Measurement of Ochratoxin A and Isolation of the fungi producing Ochratoxin A from Korean traditional fermented soybean foodstuffs

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# 전통 대두醱酵食品중에 존재하는 Ochratoxin A 생산菌 分離와 Ochratoxin A량 측정

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**ABSTRACT: Fungal ochratoxin A wes extracted and measured from the Korean traditional and fermented soybean foodstuffs (12 samples of Maeju, 28 samples of Dwangjang and 28 samples of Kangjang) collected from the whole nation wide regions. The various fungi were isolated from the foodstuffs and they were also examined whether the isolates produce ochratoxin A (OA) under the artificial conditions or not. Determinations of OA produced by the fungi were done with the antibody-attached CIA method, which was accurate or sensitive at the range of 20 pg per sample with a ninty percent recovery. Out of the 222 fungal isolates, 39 isolates produced the OA under the artificial conditions, and were identified as species of *Aspergillus, Penicillium, Paecilomyces* or other genera. The OA detected in all soybean foodstuffs was presumed to be originated from the first fermentation step of maeju.**

**KEYWORDS: Ochratoxin A, Korean traditional foodstuffs, Soybean, Fermentation, Maeju, Dwanjang, Kanjang.**

Ochratoxin A was reported to be a mycotoxin containing a kind of isocumarin and considered to toxic on the mankinds. In the studies of its mycotoxin on the experimental animals, it caused the nephrotoxicosis (Krough *et al.*, 77; Krogh, 1979), hepatogenesis (Szczech *et al.*, 1973, and teratogenesis (Hayes *et al.*, 1974), and finally disturbed the immune systems (Ciegler *et al.*, 1972; Richard *et al.*,1985). Ochratoxin A was first detected from the dairy foodstuffs (Chu *et al.*, 1978; Peska *et al.*, 1981) and the fermented products of meat and milk (Escher *et al.*, 1973; Northolt *et al.*, 1979; Bullerman *et al.*, 1984). Ochratoxin A was considered to be contaminated from various foodstuffs (not indicated) and was also speculated to be a secondary metabolite produced by the fungi. Ochratoxin A detected on the protein-foodstuffs was ware of human being's health in the forgien countries, but not in our country.

Mycotoxins was worldwide studied and reported on several foodstuffs made from the cereal or dairy products (Lee *et al.*, 1984; Dominique *et al.*, 1985). Particularly, aflatoxin was reported to be detected on a Korean traditional foodstuffs (이 등, 1988; 박 등, 1989), but a little was known about other mycotoxins on Korean foodstuffs. A Korean traditional soybean foodstuffs, especially, made from the fermented soybean cakes (maeju), was very important on the Korean diets. The fermented soybean products, maeju (the raw materials from the soybean cakes), kanjang (Soya sources), and dwanjang (Paste), were made from the soybean by the natural and traditional fermentation process. Thus, they were considered to have high

potentials to be contaminated with the fungi producing mycotoxins (이, 1988; 김 등, 1977). Therefore, the studies of mycotoxins were not reported or studied for them, yet.

Maeju is a nutrient rich cake for the fungi, as made from the cake of high protein-soybeans. Perhaps, it is considered to contain OA with other mycotoxins or other fungal metabolites. Thus, this work was subjected to measure the OA on the Korean traditional soybean foodstuffs collected from many provinces, and to isolate the fungi producing OA under the artificial conditions and contaminated with the soybean foodstuffs collected. Ochratoxin A was detected from the Korean traditional soybean foodstuffs by the immunoassay methods. The fungi producing OA were speculated to produce it under the natural systems.

## Materials and Methods

### Chemicals and reagents

The chemicals used in the immunoassay, Ochratoxin A(OA), Complete freund's ajuvent, Sephrose-4B-protein A, and microperoxidase (MP- 11) were all reagents purchaged by Sigma Chemical Company(P.O.Box 14508, St. Louis, Mo., U.S.A. 63178-9916) unless otherwise noted. Amino buthyl isoluminol (ABEI) was purchased from LKB(Wallacoy Turke, Filand). Culture media used, Potato dextrose(PD) agar and Czapek Dox(Cz) agar were products of either Difco laboratories(Detroit, MI) or BBL Microbiology Systems(Becton Dickinson and Co., Cockeysville, MD) unless otherwise noted.

### Collections of samples and preparations

The samples of Korean soybean foodstuffs, Maeju, Kanjang, and Dwanjang were collected for the houses located at several cities or provinces as shown in Table-I. All samples collected were stored in a deep freezer at −70℃. The maejus collected were in the state made in around November, 1989, and predicted to be manufactured for kanjang and dwangjang on Ferburary or March, 1990. The maejus were dried at 45℃ for 24 hrs, milled by Disc Mill, and stored in air tied polyethylene bags under the vacuum. The kanjang (liquid state) or the dwanjang (paste state) were a home made state.

**Table I.** Sources and numbers of Korean fermented soybean foodstuffs collected and used for analysis.

| Areas collected | Number of samples from the foodstuffs | | | |
|---|---|---|---|---|
| | Meju[a] | Dwanjang[b] | Kanjang[c] | Total |
| Pusan | – | 3 | 3 | 6 |
| Taejeon | 2 | 5 | 6 | 13 |
| Kwangju | 1 | 5 | 4 | 10 |
| Kunggi | 6 | 4 | 5 | 15 |
| Seoul | 3 | 11 | 10 | 24 |
| Total | 12 | 28 | 28 | 68 |

[a]Meju is composed of only soybean and naturally inoculated with several fungi for soy-sources and paste fermentations.
[b,c]Dwanjang and Kanjang collected for home made products at household level.

### Chemiluminescane immunoassay (CIA)

The measurements of OA was basically conducted by the test of antigen-antibody. The 2 mg of OA-BSA synthesized (한, 1990) was dissolved in 0.5 m*l* of 0.05M PBS (phosphate buffer pH 7.4), and emulsified with 0.5 m*l* of complete freund's adjuvant. The multifulsite intradermal injections were conducted on the back muscles of the rabbits, and boostered at the interval of two weeks. The detailed methods of antibody of OA was described by Kang (강; 1985). The antiserum collected from the above was passed through the affinity column (Sepharose-4B-protein A affinity column) and the immunoglobin G for OA purified with the eluent solution (acetate buffer, pH 4.0). Ochratoxin A binded with ABEI (OA-ABEI) was used at the 1:2000 dilutions of 0.05M PB (pH 7.4) (Kim, 1982; Han, 1990).

The OA antibody were diluted with the coating buffer (0.01 M barbital buffer, pH 9.6) at the 200 times was placed on the polystyleen star tube (Nunc, Denmark) after then, coated at 4℃ , overnight (or 37℃, 2 hr) and washed by the washing buffer (0.05 M PB-Tween 20), three times. After then, ochratoxin A-ABEI was reacted to Ig G at 37℃ for two hours, washed with the buffer(washing buffer: PBtween 20) and the reaction of OA-ABEI to Ig G stopped with the addition of 0.2 m*l* of 5 NaOH and with incubations at 60℃ for

1 hour. After then, the reacted tube was placed at the room temperature for 30 min. and then was put in to Luminometer. That tube was automatically injected 0.3 ml microperoxidase (1:100) and 0.3 ml $H_2O_2$ (35%:1:100). The amounts of Luminoscence were measuerd by Luminometer(model clinilumat LB 9502, Schwerzwald) for 4 seconds.

**Extractions of Ochratoxin A**

Ochratoxin A (the samples mentioned in the above or the fungus cultured agar) was extracted with chloroform, filtered by anhydrous sodium sulfate ($Na_2SO_4$) column, and concentrated by the rotary evaporator (Tokyo Rikiaai, Japan) at 40℃.

The OA concentrated was dissolved in the methanol and diluted with the solution of 0.05 M sodium phosphate buffer (pH 7.4) until the concentrations of methanol was less than 1 percent.

**Cultures and Isolation of fungi**

PD or Cz agar was autoclaved and its pH was adjusted with the solution of 10% tartaric acid. The samples of the upper mentioned were ten times diluted with the sterilized 0.9% NaCl solutions and plated on either agar under the flow of the sterile air. Fungi were isolated from the single colonies cultured on the PD agar at 25℃ was based on the colors of colony and morphology of conidia and transferred into the slants for storage. The slide cultures of each fungal isolate were prepared on the 2% Malt agar or PD agar, to observe the natural state of conidiospores or conidia attachments (Kendrick and Carmichael, 1973, Fassatiova, 1986). Several pieces of agar containing the mycelia or cells of the yeast were placed on the slide glasses and smashed with the cover glass. Then, the mycelia were observed under the light microscope (80X, 320X, 800X), and also observed, sometimes, after stained with 0.05% lactophenol tryphan blue. The specimens of the yeast isolates made by the slide cultures were stored at Dr. Lee, Sang-Sun's laboratory in Korea National University of Education.

The identification of the isolated fungi was conducted at the level of genus by the taxomonical keys described by Barron(1968), Fassatiova(1986), and Kendrick and Carmichael(1973).

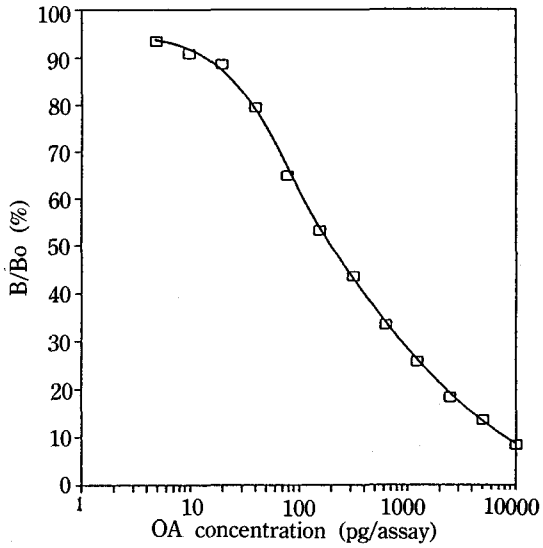


**Fig. 1.** Standard curve for OA in solid-phase Chemiluminescence Immunoassay (CIA).

## Results and Disscussions

**Standard curve of Ochratoxin A**

The 200 μl solution of Ochratoxin A antiserum diluted were coated in the polystylrene star tubes and competely reacted with the Ochratoxin A attached with the tracer (Ochratoxin A-ABEI). Fig. 1 is a standard curve of Ochratoxin A-ABEI with the sensitivity of 20 pg/assay ($2\times10^{-11}$g per sample) and was also done by Pestka *et al.* (1981) and Han (1990). The chemical method for mycotoxin determination required the great amounts of samples and interfared with other compounds. Also, a great amounts of Ochratoxin A was lost throughout the complicated process of extraction (Gregory ., 1981). However, the CIA used here was considered to be simple without any extract procedure but also senstive in the low concentrations of Ochratoxin A.

**Recovery test**

The Ochratoxin A was added to the agar plate with 1 or 2 μg per dish, artificially mixed with shaking, and placed for 1 hour. After then, the agar plate was mixed with the 100 ml of methanol and Ochratoxin A was directly extracted from the agar. The methanol solution containing the Ochratoxin A was diluted until less than a percent of

**Table II.** The colonies or isolates producing ochratoxin A and collected from various Korean tradititonal soybean fermented foodstuffs.

| Foodstuffs[a] | Number of samples | Number of fungal isolates (producing OA[b]/collected)[c] | Fungal isolates[d] |
|---|---|---|---|
| Meju (M-series) | 10 | 19.27% (16/83) | M12, M13, M26, M35, M36, M48, M49, M66 M611, M612, M72, M74, M81, M84, M88, M94 |
| Dwanjang (D-series) | 10 | 18.30% (13/71) | D11,D17,D18,D21, D31, D46, D49, D54, D62, D62, D64, D73, D74, D81 |
| Kanjang (K-series) | 10 | 14.70% (10/68) | K11, K15, K16, K25, K27, K31, K36, K43, K46, K49 |
| Total | 30 | 17.56% (39/222) | 39 isolates |

[a]Korean traditional soybean foodstuffs organized by the process.
[b]Total number of fungal isolates producing Ochratoxin A on PD agar.
[c]Number of fungal isolates collected from various soybean foodstuffs.
[d]Fungal isolates from Meju (M-series), Dwanjang (D-series) and Kanjang (K-series)

methanol, and checked for Ochratoxin A determination. Ochratoxin A determination, when 1.0 μg of Ochratoxin A added to the agar, showed that the recovery rate was 92.3± 6.2% at the 95% confidence level, and when 4 μg of Ochratoxin A added, that the recovery rate was 91.1± 5.4% at the 95% confidence level. The CIA was a direct determination, especially regardless of chemical extraction between the color determination and samples, so that the recovery rate was high. The CIA method employed for Ochratoxin A determination was considered to be reliable and practical to determine the Ochratoxin A from the various samples and to reduce the variations from the experimental error. The Ochratoxin A was determined from the fermented soybean foodstuffs, maeju, dwanjang and kanjang, but not indicated here. The soybean foodstuffs containing Ochratoxin A were selected and employed for the further works. The various ten soybean foodstuffs were confirmed to be contaminated with Ochratoxin A by the CIA determination. The fungi were isolated from each soybean foodstuff contaminated with Ochratoxin A and marked as shown in Table II. The fungal isolates were marked as the orignins of fungi; The M, D and K marked isolates were originated from Maeju, Dwangjang, and Kangjang, respectively.

**Isolations of the fungi producing Ochratoxin A**

Tartaric acid (14 m*l*/l, War, 1950) and streptomycin (30 mg/l, Beever *et al.*, 1970) were added to PD agar for contaminations of the bacteria. The 10 g of the mashed samples or liquid samples were weighed and mixed with the 90 m*l* of sterile saline solution. The diluted saline solution containing the samples was shaked and placed on the PD agar by the plate counting method. A single colony appeared on PD agar was isolated as based on the morphology and color of fungi, transferred into other PD agar until a kind of colony was obtained, and stored on the PD agar slant for identifications. The 83 colonies of fungi were obtained from the maeju, 71 colonies from Dwangjang, and 68 colonies from Kangjang. Total 222 colonies of fungi were isolated from the Korean fermentated soybean foodstuffs. Each colony or isolates of fungi were inoculated on PD agar, again and incubated at 25℃ for ten days. PD agars cultured were harvested and mechanically mixed with chloroform for the determinations of OA (Lee, 1984). Out of 83 isolates, the sixteen isolates were determinated to produce OA in the foodstuffs of maeju, vice visera. Less isolates producing OA collected from kangjang or dwangjang than maeju were found. Also, less isolates producing OA were found in kangjang or Dwangjang than in maeju were found (Table-II). The detail determinations of OA were conducted from the isolate cultured

**Table III.** Numbers of fungal isolates producing Ochratoxin Aa).

| Amount of Ochratoxin A produced in two petridish (ng)[b] | Numbers of isolates producing Ochratoxin A |
|---|---|
| 20×100 | 13 |
| 100×200 | 18 |
| 200×500 | 6 |
| 500×2000 | 2 |

[a]Fungal Isolates incubated on PD agar at 25 for 10 days.
[b]Ochratoxin A extracted from fungal two isolate grown PD agars and determined by CIA

PD agar in the two petri-dishes as shown in Table-III. The eighteen isolates were found to produce OA at the ranges of 100 to 200 μg per two petri-dishes. Also, two isolates were determined to produce OA at the high level of 500 to 2000 mg. Conclusively, the isolates in Table-III were considered to produce OA in the natural fermentation of soybean foodstuffs.

**Identification of fungi producing Ochratoxin A**

The fungi were observed by naked eyes or under a microscope. The isolates were cultured on PD agar or CY agar at the room temperature for two or three days, to identify them if necessary. The detail observations of condiogenesis of the isolates collected were conducted by the slide cultures on Malt or PD agar and by staining lactophenol blue. By naked eyes, the color, growth habits, agar color (screted by the isolates) of the isolate colonies were recorded, to help the identification. The conidiogenesis of the isolates (various types of spore), formations of sterigmata or metulae, were observed under high resolution microscope, ususally 1000 times. Here, the identifications of the isolates were tried in best under the limited conditions of our laboratories, but limited to the level of genus, because the limitation of descriptions of the isolates.

Several features and colors of condia indicated under microscope were those of conidiogenesis or colors traditionally known as keys of Deuteromycotina, Saccardoan classification. The isolates,

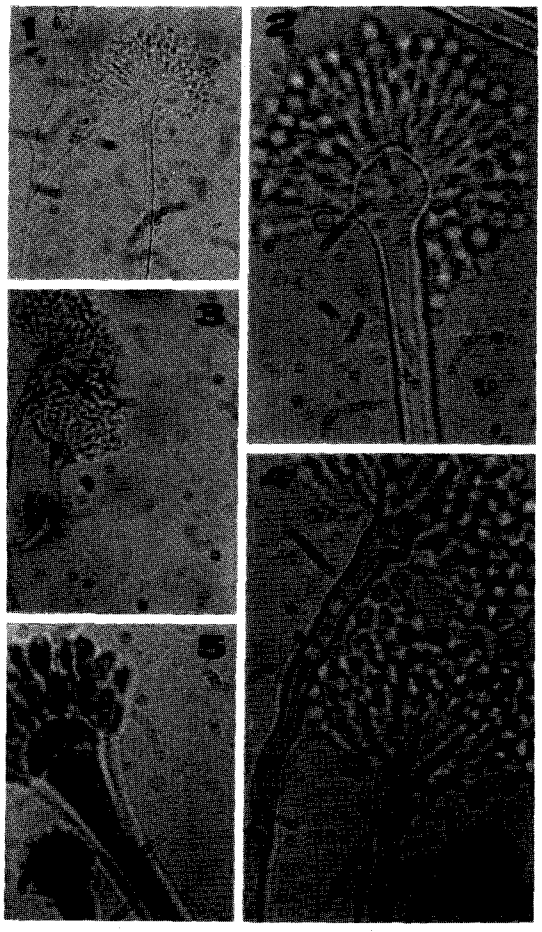

**Fig. 2.** *Aspergilli* collected from the soybean foodstuffs : 1) 8×25 from dwanjang, 2) 8×100 from dwanjang and maeju, 3) 8×40 from maeju, 4) 8×100 from dwanjang and maeju, and 5) 8×100 from maeju magnified under a light microscope.

after the slide cultures observed, were identified at the level of genus, as using the keys made by Barron(1968), Kendrick and Carmichael(1973), von Arx(1981), and Fassatiova(1986). Phialospores were observed in the slides of *Penicillium, Paecilomyces* and *Aspergillus* (Table-IV and Fig. 2,- 3 and - 4). The chain of basiptal phialospores was observed to be covered with the sheath in the isolate of the genus of *Pasecilomyces* (Fig. 4-1 and 4-3) and it was consistent with the description made by Kendrick and Carmichael (1973) and by Samson (1974). A blastospore with acropetal chains defined was found in the slide of Cladiopo-

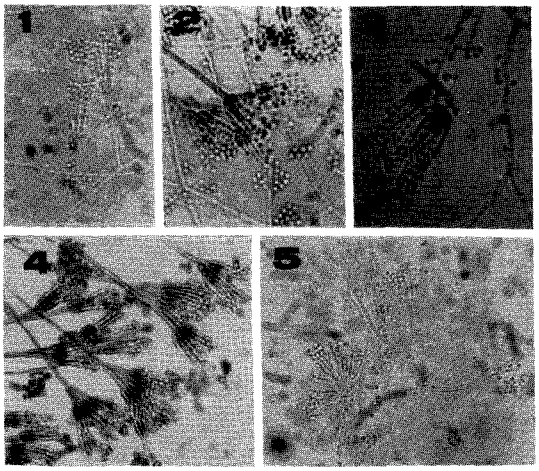

**Fig. 3.** *Penicillia* collected from the soybean foodstuffs : 1) 8×40 from dwanjang, 2) 8×40 from dwanjang and maeju, 3) 8×40 from maeju, 4) 8×25 from dwanjang and maeju, and 5) 8×25 from maeju magnified under a light microscope.

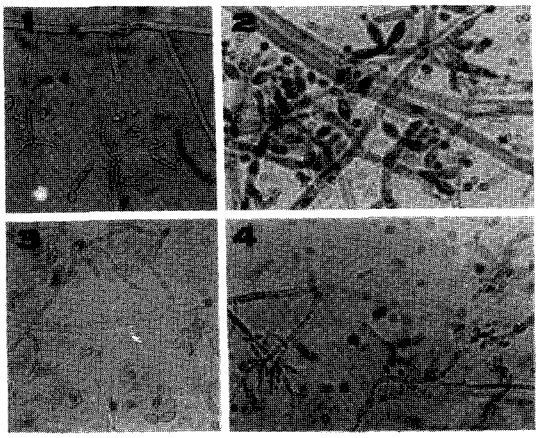

**Fig. 4.** The various condia of 1) 8×40 and 3) 8×100 both Paecilomyces from dwanjang and kanjang, 2) 8×100 Trichoderma from maeju, and 4) 8×40 Cladiosporium from dwanjang and maeju.

rium (Fig. 4-4), but phialospores of *Trichoderma* (verticils, Fig. 4-2).

All isolates producing Ochratoxin A under the artificial conditions were identified as shown in Table IV. Several types of *Aspergillus* and *Penicillium* were observed among the isolates collected as shown in Table IV. Detailed identifications of them were beyond the objects of this experiments and would be in the next work. Various conidia of the isolates of *Aspergillus* were observed as shown in Fig. 2. The biseriate stigmata on vesicles of conidia were hardly found under the light microscope, but a few of them were clearly observed. The isolates belonged to the genus of *Aspergillus* were tried to be identified on basis of Raper and Fennell's (1973) description. *Asp. vericolor, Asp. orchacous,* and *Asp. fumigatus* were identified (Fig. 2), but were not compared with the standard species supplied by ATCC or other identified species. The isolates belonged to the genus of *Penicillium* were tried to be identified on basis of Pitt's (1979) description as shown in Fig. 3. *Pen. restrictium* was identified as based on the type of *Aspergilloide vesicles* (Fig. 3-1), but the other penicillia, the species of *terberticillate, beverticillium,* and *furcatum* groups were also found under the light microscope. Most of the identified or unidentified isolates were considered to have a dry and airborne spores in their habit. In conclusion, the most serious fungi contaminated in the fermented soybean foodstuffs were the species of *Aspergillus* (7/39) or *Penicillium* (20/30).

**Soybean foodstuffs**

Maeju is considered to be a soybean cake, so called "koji", inoculated with the natural flora of fungi and bacteria. Also, it is considered to be biochemically composed of mainly protein. It was considered to be contaminated with the air borne fungi, which are capable of utilizing soy protein. Perhaps, it is a traditional food containing protein and protein hydrolyzing enzymes produced by the fungi. Various and many isolates found in the fungal flora of maeju was consistent with our results (Table IV). It was considered to be contaminated with the air borne fungi (*Penicillia* and *Aspergilla*). Maeju was mixed with salted water and after then, anaerobically inside and aerobically outside fermented for a month. Kangjang was made from maju mixed with salted water as a liquid part and dwangjang as a solid part. Less numbers of fungal isolates found from both than from maeju were consistent with the process of fermentations. Therefore, 17.56% fungal isolates collected produced OA on PD agar. Regarding of this results, Ochratoxin A found in the various soy food-products, Ka-

**Table IV.** Identification of Ochratoxin A producing fungal isolates[a].

| Genera | Numbers of fungal isolates | Foodstuffs[b] (Numbers of isolates) |
|---|---|---|
| *Aspergillus* | 7 | Meju (3), Dwanjang (4), Kanjang (1) |
| *Pencillium* | 20 | Meju (10), Kanjang (5), Dwanjang (4) |
| *Paecilomyces* | 4 | Meju (1), Kanjang (4) |
| *Cladiosporium* | 1 | Meju (1) |
| *Phialotubus* | 1 | Meju (1) |
| Unidentfication | 6 | Dwanjang (3), Kanjang (2) |
| Total | 39 | Maeju (16), Kanjang (10), Dwanjang (13) |

[a]Fungal isolates identified at the level of genus using the keys made by Barron (1968), Kendrick and Carmichael (1973), von Arx (1981), and Fassatiova (1986).
[b]( ) indicated the numbers of isolates collected from the different soybean foodstuffs.

ngjang or Dwangjang would be originated from Maeju. For further speculation, Ochratoxin A would be produced by the air borne flora of fungi. Ochratoxin A contaminated in kanjang or dwanjang was speculated to be originated from the maeju, the natural process of soybean cakes. The natural fermentation of maeju was considered to be a very important process, to be related with OA or other mycotoxins.

## 摘 要

한국내에서 재래적인 방법에 의하여, 생산, 시판되고 있는 전통 발효식품인 메주 (12종), 된장 (28종) 및 간장 (28종)을 전국에서 수거하였다. 이들로부터 OA 생성하는 fungi를 분리하였다. 분리된 균으로 다시 Ochratoxin A 생성 유무를 관찰하였으며, 개괄적인 균 동정도 시도하였다.

1. Ochratoxin A를 定量調査한 표준곡선의 作成結果, 감도 (Sensitivity)는 20 pg/tube 수준 이었다.

2. 본 실험에서 이용한 OA의 분석 방법에서 OA의 回收率은 90% 이상 이었다.

3. 각 試料에서 分離해낸 222 fungi 중 Ochratoxin A를 생성하는 것은 39 isolates로 나타났으며, 이 중 대부분이 *Aspergillus* 속, *Penicillium* 속 그리고 *Paecilomyces* 속 이었다.

4. 전통 발효식품인 메주,된장,간장내의 곰팡이중 Ochratoxin A 생성곰팡이의 생성률은 각각 19.27%, 18.30%, 14.70% 으로 나타났으며 0.2g / 2 petridish 이상의 OA 생성률은 20.5% (8/39)로 나타났다.

또한 Ochratoxin A 생성에 대한 몇가지 추론도 함께 하였다.

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