Review on Citrinin: Production, Effect of Some Plant Extracts and Gene Involved in its Biosynthesis

Alaa Mostafa Abou-Zeid
Botany Department, Faculty of Science, Tanta University, Tanta, Egypt

Introduction

Citrinin is the one of the well-known mycotoxins, which is possibly spread all over the world. It is originally isolated from Penicillium citrinum and now produced by a variety of other fungi. Citrinin was produced in liquid potato-dextrose medium and determined by TLC and HPLC. Extracts of some plants reduce or inhibit citrinin production. Some of the genes involved in Citrinin biosynthesis were discussed.

Physicochemical Properties

Citrinin \([C_{14}H_{16}O_5]\), IUPAC: (3R, 4S)-4, 6-dihydro-8-hydroxy-3, 4, 5-trimethyl-6-oxo-3H-2-benzopyran-7-carboxylic acid; CAS No.: 518-75-2 (Figure 1), is an acidic lemon-yellow crystal with maximal UV absorption at 250 nm and 333 nm (in methanol), melting at 172°C [1]. It is sparingly soluble in water but soluble in dilute sodium hydroxide, sodium carbonate, or sodium acetate; in methanol, acetonitrile, ethanol, and most of other polar organic solvents [2].

It is a mycotoxin originally isolated from Penicillium citrinum. It has been found to be produced by a variety of other fungi (Aspergillus niveus, Aspergillus ochraceus, Aspergillus oryzae, Aspergillus terreus, Monascus ruber, Monascus purpureus and Penicillium camemberti) which are found or used in the production of human foods, such as grain, cheese, sake and red pigments. Citrinin has also been found in commercial red yeast rice supplements [3]. As one of mycotoxins, citrinin possesses antibiotic, bacteriostatic, antifungal and antiprotozoal properties. While it is also known as a hepato-nephrotoxin in a wide range of species [4-6].

Jackson and Ciegler [7] reported that heat degrades citrinin, but toxicity is retained in a fraction that is chloroform soluble and NaCHO insoluble. This would agree with Chu’s [8] findings that antibiotic power is retained after autoclaving. Heat had caused decarboxylation of the citrinin to a compound such as decarboxycitrinin (Figure 2a), which is a natural metabolite of P. citrinum [2], or decarboxyhydrocitrinin (Figure 2b).

It is a quinine methide with two intramolecular hydrogen bonds [1]. Citrinin crystallizes in a disordered structure, with the p-quinone and o-quinone two tautomeric forms in a dynamic equilibrium in the solid state (Figure 3).

Yao et al. [9] found that investigation of a microbial fermentation organic extract of Penicillium sp. H9318 led to the isolation of a new isoquinolinone alkaloid, (5S)-3,4,5,7-tetramethyl-5,8-dihydroxyl-6(5H)-isoquinolinone [1], along with four known citrinin derivatives [2-5] (Figure 4). Citrinin [2] exhibited significant inhibitory activity against Streptomyces 85E in the hyphae formation inhibition (HFI) assay, while compounds 1, 3-5 were not active when tested at 20 mg/dl in the HFI assay.

Cultures Conditions of Citrinin

Citrinin was produced in liquid potato-dextrose medium (PD) as described by [10]. Inocula containing 10^5 spores of each citrinin producing isolates of Penicillium citrinum were added to PD medium (3 mL) in 25 mL flasks (20x100 mm) and incubated at 26 ± 0.5°C for 21 days. Citrinin was extracted from cultures three times with 10 ml of chloroform, treated with anhydrous sodium sulfate, filtered and evaporated to dryness [11]. Residues were dissolved in chloroform (0.1 mL) [12].

Also Hajjaj et al. [13], described the production of citrinin as follows: A strain of Monascus ruber (ATCC 96218) was grown at 28°C in a chemically defined medium as described by. A suspension of 10^6 spores was used to inoculate a 1-liter baffled Erlenmeyer flask containing 200 ml of glucose medium. The presence of red pigments and citrinin in the culture broth was determined.

*Corresponding author: Alaa Mostafa Abou-Zeid, Botany Department, Faculty of Science, Tanta University, Tanta, Egypt, E-mail: abouzeid_alaa@yahoo.com

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Citrinin Determination

Citrinin was isolated from the medium by filtration of the mycelium cultures on M14 membranes (pore size of 0.8 μm). The filtrate was lyophilized, re-suspended in 60 ml of water, and extracted three times with water saturated with n-butanol. The organic phase was dried and vacuum concentrated, and the residue was dissolved in 50 ml of acidified water (pH 2.0). This solution was treated twice with 120 ml of ethyl acetate, and the retained organic phase was extracted twice with 150 ml of 0.4% NaHCO₃. The aqueous phase was adjusted to a pH of 3.0 with HCl and again extracted twice with 120 ml of ethyl acetate. The organic phase containing citrinin was evaporated to dryness and resuspended in a minimal volume of water. The toxin was isolated by thin-layer chromatography in chloroform-methanol-water (65/25/4, vol/vol/vol), and the band containing the toxin was solubilized in chloroform which was evaporated. Spectra were referenced internally to the solvent for ¹³C NMR and to trimethylsilyl for ¹H NMR. Minimization of the relative molecular energy of intermediates 2 and 3 (Figure 5) was carried out with the molecular mechanics programs Biosym and Discover on a Silicon Graphics machine. It was found that the production of citrinin started after 45 h of cultivation and did not appear to stop when the glucose from the medium was consumed [14]. The citrinin concentration was 6.5 μg/ml.

These experiments confirmed that citrinin arose from the polyketide pathway, by a route apparently similar to that found in P. citrinum and A. terreus [15,16]. However, they found that the spectra from labeled citrinin were strikingly different from those obtained for P. citrinum and A. terreus, incubated under the same conditions. The occurrence of a tetraketide as the precursor for both citrinin and red pigments may account for the differential production of these two polyketides during the growth of M. ruber (Figure 6).

Mossini and Kemmelmeier [12] determined the citrinin as follows: reported that citrinin standard was prepared in ethanol (1 mg/mL) and stored at 4°C [11]. Extracts (dissolved in chloroform) and standard underwent TLC on 20 x 20 cm Aluminium plates (Silica gel 60 – G.), with toluene-ethyl acetate-formic acid (6:4:0.5, v/v). After development the plates were exposed to 365 nm ultraviolet light (UV). Citrinin appears as a fluorescent yellow spot. The phenolic group in citrinin, estimated by Folin-Phenol reagent [17] gave a linear relationship with concentration over the 5-100 μg/mL range. HPLC analysis was performed to confirm spectrophotometric results. Residues were dissolved in an appropriate volume of mobile phase (1 mL for all samples), filtered through a 0.45 μm disposable syringe filter prior to injection into the chromatograph. Aliquots (20 μL) were injected on HPLC column and analyzed. Comparison of sample retention times with that of the standard identified the presence of citrinin in the samples. The relationships between peak height and area and the amount injected were linear over the ranges 2.5-50 ng. The citrinin production by the three isolates (K1, K4 and k8) of P. citrinum was 2.17 x 10⁻², 4.25 x 10⁻² and 6.3 x 10⁻² μg respectively as measured spectrophotometrically.

Also Reddy et al. [18] extracted and determined citrinin as follows:

The culture filtrates (10 ml) of P. citrinum, isolated from rice grain, was used for extraction and estimation of citrinin. The citrinin was extracted three times with chloroform (1:1 v/v), pooled and concentrated in vacuo at 40°C using a rotary evaporator. The crude extract was diluted in minimum amount of chloroform (2 ml) and citrinin was estimated by thin layer chromatography (TLC) according to Razak et al. [19] with minor modifications. Briefly, different volumes (1 to 5 μl) of sample extracts were applied to precoated TLC plates (TLC Silica gel 60 F254, Merck, Germany) along with standard (containing citrinin at 0.5 μg/ml). The plates were developed in toluene/ethyl acetate/formic acid (6:4:0.5 v/v) in glass tanks covered with aluminum...
foil [12]. After development, the plates were dried and observed under long wavelength (365 nm). Citrinin appears as a fluorescent yellow spot. The intensity of the sample spots was compared with that of the standard spot. The citrinin concentration was 9.2 µg/ml.

### Effects of Some Plant Extracts on Citrinin Production

**Neem leaf extract (NLE)**

Dried leaves of *Azadirachta indica* A. Juss (Meliaceae) were extracted by maceration in distilled water (100 g/L) and stirred for five hours in the dark, at room temperature. At the end of the extraction, the material was sieved through Whatman 1 filter paper, freeze-dried, and preserved in dark flasks. A 10% aqueous extract of the residue was prepared and used [20]. Treatments in four replicates consisted of 10% freeze-dried aqueous NLE at concentrations 3.12, 6.25, 12.5, 25 and 50 mg/mL added to the PD, before autoclaving and inoculation [12]. After 21 days, quantitative determination of the extracts from liquid culture media demonstrated inhibition of citrinin production by three isolates of *P. citrinum* on media with NLE. Neem extracts of 3.12 mg/mL reached 87.16% inhibition on K4 and 85.86% inhibition on K1 and 94.86% inhibition on K8 citrinin production at NLE 6.25 mg/mL (Figure 7).

**Aqueous extracts of five medicinal plants**

Leaves of five medicinal plants (*Andrographis paniculata*, *Cymbopogon citratus*, *Eurycoma longifolia*, *Kaempferia galanga* and *Orthosiphon aristatus*) were collected and washed under tap water. Then the leaves were dried in hot air oven at 60°C for 4 days and ground to made powder to pass through 20 mesh sieve. Ten grams of ground powder were shaken in 100 ml distilled water at 200 rpm for 5 h at room temperature [19]. The supernatant was collected and passed through 0.22 µm membrane filter and stored at -20°C until use. Various concentrations (2.5, 5.0, 7.5 and 10.0 mg/ml) of aqueous plant extracts were added to cooled liquid broth. A 10 µl amount from suspension contained 10⁵ spore/ml of fungus was inoculated in each flask and shaken at 200 rpm for 10 days at 25 ± 2°C. The control contained PDB broth and 10 µl of fungal suspension [18]. It was found that all plant extracts effectively reduced the citrinin production ranging from 42.3 to 91.3% at 10 mg/ml concentration in liquid media. Among the plant extracts tested, *C. citratus* effectively inhibited the citrinin production by *P. citrinum* ranging from 22.8 to 91.3% followed by *A. paniculata* ranging from 8.6 to 83.6% at all concentrations tested. Other plant extracts showed less reduction ranging from 42.3 to 54.3% even at higher concentration (10 mg/ml).

### Gene Involved in Citrinin Biosynthesis

During the biosynthesis of the secondary metabolites like citrinin, many biosynthetic enzymes are required and should function coordinately in the synthesis of these structurally complex metabolites, many of which are polyketides. Polyketides, such as pigments and mycotoxins, are structurally diverse and often complex compounds that are at least partially synthesized by multifunctional enzymes called polyketide synthases (PKSs). The genes encoding these enzymes have often been reported to localize in an adjacent region or to form a gene cluster [21], similar to the situation of biosynthetic gene clusters for secondary metabolites in prokaryotic actinomycetes.

In Shimizu et al. [22] study, a PKS gene for citrinin (CT) (*pksCT*) was cloned from *M. purpureus*. They reported that PCR with primer pair KS and LC5c (Figure 8) yielded a single distinct product, and the PCR product (430 bp) was very close to the expected size, 420 bp, calculated from the sequences of other fungal PKSs. The deduced amino acid sequence was 49%, similar to the aflatoxin biosynthetic PKS from *A. parasiticus* and 43% similar to the bikaverin biosynthetic PKS from *G. fujikuroi*. These similarities suggested that the PCR fragment was...
part of a PKS gene responsible for the biosynthesis of multi-aromatic-ring polyketides.

The complete PKS gene (7,838 bp; DDBJ accession no. AB167465) encoding a 2,593-amino-acid protein (Figure 9) was obtained from the *M. purpureus* genome by three rounds of colony hybridization by using probe A to identify a BamHI (1)-BamHI (2) fragment and an EcoRI-SalI fragment and probe B to clone a KpnI (2)-KpnI (3) fragment (Figure 9).

In Shimizu et al. [23] study, they cloned the genes in the vicinity of *pksCT* to obtain new genes involved in CT biosynthesis. An activator gene essential for the efficient production of CT was found in the upstream region of *pksCT*, and they demonstrate that an extremely low-CT producer can be created by disrupting the gene.

The transcription patterns of the five ORFs were examined by RT-PCR with RNA samples prepared from mycelia cultivated for 2, 4, or 6 days under CT production conditions. *pksCT* was transcribed from the 2-day cultivation, at which point CT production started in the wild-type strain (Figure 10A). Similarly, the transcripts of the four other plausible genes (*orf2*, encoding a regulator; *orf3*, encoding an oxygenase; *orf4*, encoding an oxidoreductase; and *orf5*, encoding a transporter) were detected from the 2-day cultivation, whereas no transcription of *orf1* was detected [23].

Results suggested that, as in the case of AflR in the aflatoxin biosynthetic cluster [23,24], the 2 open reading frames *orf2* product (designated CtnA for being the first factor related to CT biosynthesis other than PksCT) acts as an activator at least on *pksCT* and (*orf5*) transcription. This occurs probably through binding to specific DNA sequences in the upstream region of each of the two genes, although no definite conserved sequence was discovered in the promoter regions of *pksCT* and *orf3*.

Sakai et al. [25] found that in the region flanking *pksCT*, there are four more ORFs in addition to *ctnA*. The estimated gene products of these ORFs showed high similarity to a dehydrogenase (orf1), an oxygenase (orf3), an oxidoreductase (orf4), and a transporter (orf5), respectively (Figure 11A). Therefore, all four genes, including *pksCT* and *ctnA*, appear to constitute a CT biosynthetic gene cluster. Because the putative CT biosynthetic cluster is relatively small (around 20 kb) it provided a suitable cosmid insert.

Characterization of *A. oryzae* transformants with CossCT containing the CT biosynthetic gene cluster following a partial BamHI digestion, the fragment containing the 20-kt CT biosynthetic cluster with sufficient flanking regions (4 kb on one side and 9 kb on the other) was recovered from SuperCos CT-1 and was transferred into the *C. Aspergilli-E. coli* shuttle vector creating CossCT. CossCT was then transformed into the heterologous host *A. oryzae* NS4. The transformants were selected on SC medium and their genotypes were...
confirmed by Southern blot using part of orf4 as a probe (Figure 11A and 11B). They verified that two transformants (strains 1-1 and 16-2), each contained the entire putative CT biosynthetic gene cluster, although strain 16-2 displayed an additional band with a lower molecular weight than the expected size of 16.1 kb. This band likely arose from a truncated form of the cluster (Figure 11B).

Sakai et al. [25] concluded that introducing additional copies of an activator gene (ctnA), controlled by the Aspergillus nidulans trpC promoter, into the citrinin-cluster-containing transformants enhanced the transcription of all the genes in the cluster and resulted in an almost 400-fold higher citrinin production compared to that of the parental transformant.

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