Determination of the Mycotoxin Cyclopiazonic Acid by Enzyme Immunoassay

Sabine Hahnau and Elmar W. Weiler*

Lehrstuhl für Pflanzenphysiologie, Ruhr-Universität Bochum, Universitätsstrasse 150, D(W)4630 Bochum, Germany

Antisera highly selective for the mycotoxin cyclopiazonic acid (CPA) were raised in rabbits. A competitive enzyme-linked immunosorbent assay (ELISA) for CPA, with use of alkaline phosphatase as tracer enzyme, was developed. The assay measures from 30 pg to 2 ng of CPA and showed an interassay, between-day variability of 14% and an intraassay variability of 3.7%. An efficient selection technique was devised that allows detection of CPA, even at low levels, in fungal strains growing as agar surface cultures. No precleaning of the samples is required. The technique allows efficient monitoring for fungal isolates producing CPA.

Cyclopiazonic acid (CPA, 1) is an indole tetramic acid metabolite of *Penicillium* and *Aspergillus* species. CPA is acutely toxic to rats (LD_{50} 2.3 mg/kg of body weight) when adminstered ip (Purchase, 1971) and produces hyperaesthesia, convulsions, and neurotoxic effects leading to death within 30 min. The toxin produces necroses in liver, spleen, pancreas, and kidney tissue and is rapidly distributed in the organism. Given orally, however, CPA is much less toxic (rat LD_{50} 36 mg/kg of body weight; Purchase, 1971). CPA is a strong inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase in vitro and presumably interferes with the ATP-induced conformational change related to Ca^{2+} translocation (Seidler et al., 1989).

Among the fungi reported to produce CPA are *Aspergillus flavus* (Gallagher et al., 1978) and *Penicillium griseofulvum* (de Jesus et al., 1981), formerly described as *Penicillium cyclopium* (Holzapfel, 1968). These species are frequently encountered on stored grain, cereal products, and groundnuts. Another producer of CPA is *Penicillium camemberti*, a species important in food technology as starter culture for the production of white mold fermented cheese (Le Bars, 1979). CPA production is a common feature of *P. camemberti* (Geisen et al., 1990, and refs cited therein), and up to 5 mg of CPA/kg of cheese was found in earlier studies, especially at elevated storage temperatures (Still et al., 1978).

The monitoring of CPA contamination of food and the selection of toxin-free industrial starter cultures of *P. camemberti* are hampered by the lack of a simple-to-perform yet sensitive and reliable analytical technique with a large sample-processing capacity. The analysis of CPA by HPLC is rather laborious, although sensitive [e.g. Goto et al. (1987)], while TLC separation combined with colorimetric determination by spraying with Ehrlich's reagent allows multiple samples to be processed simultaneously but provides rather limited sensitivity and selectivity (Le Bars, 1979; Geisen et al., 1990).

To devise an improved analytical procedure both for the quantitation of CPA and for the efficient screening for the absence or presence of the toxin, we have (a) raised CPA-specific antibodies, (b) devised a competitive enzyme-linked immunosorbent assay (ELISA) which allows the detection of picogram quantities of CPA, and (c) designed a simple technique for screening fungal isolates for CPA production. Our initial results confirm the production of CPA by white-mold fungi isolated from cheese samples.

* Address correspondence to this author.

MATERIALS AND METHODS

Chemicals. Cyclopiazonic acid was obtained from Sigma Chemical Co.; tenuazonic acid, fumitremorgin B, and penitrem A were kind gifts of Dr. Fink-Gremmels, Kulmbach, Germany.

Fungal Material. The following fungal reference strains were used (maintenance culture medium given in parentheses; for composition, see below; ATCC, American Type Culture Collection; CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; Sp, culture collection of the Federal Centre of Meat Research, Kulmbach, Germany): *Aspergillus caespitosus* ATCC 11256 (ME); *Aspergillus flavus* ATCC 36182 (MEN); *Aspergillus fumigatus* CBS 192.65 (MEN); *Penicillium commune* Sp 376 (MEN); *Penicillium camemberti* Sp 438 (MEN); *Penicillium camemberti* Sp 1083 (MEN); *Penicillium ochrachloron* CBS 338.59 (MEN); *Penicillium paxilli* CBS, 547.77 (MEN); *Penicillium puberulum* Sp 123 (MEN); *Penicillium simplicissimum* CBS 362.48 (MEN); *Penicillium verrucosum*, var. *album* CBS 343.51 (MEN); *Penicillium verrucosum*, var. *cyclopium* CBS 341.59 (MEN); *Penicillium verruculosum* ATCC 24640 (MEN). Blue-mold strains isolated from commercial blue-veined cheeses were designated SK codes and maintained on CMA; white-mold isolates from white surface mold cheeses were designated WSK codes and maintained on MEN (WSK 1, 2, 4, 8) or CMA (WSK 5, 6). The culture media were as follows: ME medium; ATCC medium 325; MEN medium; ATCC medium 324; CMA medium; 20 g of glucose and 20 g of cornsteep were made up with double-distilled H_2O to 1 L, the pH was adjusted to 6.5, and 15 g of agar was added. Maintenance cultures were transferred to fresh agar-solidified medium every 4 weeks and were grown at 25 °C in darkness.

Synthesis of Immunogens and Enzyme Tracers. While a CPA–BSA conjugate is commercially available (Sigma), the type of linkage of the hapten to the carrier is not known. Therefore, immunogenic conjugates of CPA to BSA were synthesized for this study using different coupling reactions. CPA was linked to bovine serum albumin (BSA) by the Mannich reaction. The toxin (4.8 mg, 14.3 μmol) was dissolved in 0.96 mL of 0.1 M $NaHCO_3$/methanol (1:1 v/v). BSA (10 mg, 0.15 μmol) was dissolved in 0.2 mL of double-distilled H_2O, and then 0.4 mL of 3 M sodium acetate was added. Formaldehyde (0.2 mL of a 37% solution) was added dropwise over a period of 1 min at room temperature (RT) with stirring. Stirring was continued for 10 min. The CPA solution was added over a period of 5 min to the reaction mixture. The final pH of the reaction mixture was 8.5. Stirring was continued for 16 h at RT. The reaction mixture was then dialyzed for 3 days against 10 L of deionized H_2O per day. The dialysate was lyophilized and yielded the conjugate in quantitative yield (11.1 mg) as a fluffy white product. This was

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redissolved (1 mg/mL) in phosphate-buffered saline, pH 7.4 (6 mM K_2HPO_4, 2 mM NaH_2PO_4, 150 mM NaCl, PBS) and stored aliquotted at −18 °C.

For comparison, CPA was linked to BSA through an *O*-carboxymethyloxime bridge. CPA (7.5 mg, 22 μmol) was added to 4.5 mg (41.3 μmol) of aminooxyacetic acid (hydrochloride salt) in 1.5 mL of dry ethanol/pyridine (1:1) which led to the instantaneous conversion of the toxin to the oxime product. The solvent was evaporated, and the residue was redissolved in 0.7 mL of methanol and chromatographed on three silica gel plates (silica gel 60 F_{254} 0.2 mm, Merck) with methanol–benzene (2:1 v/v) as mobile phase. The UV-absorbing bands on each plate (R_f 0.84) were eluted with methanol. After concentration of the eluate, the preparation was rechromatographed on three silica gel plates [solvent, ethyl acetate–methanol (4:1 v/v)]. The UV-absorbing material (R_f 0.21) was eluted with methanol. The solvent was removed. The residue (8.1 mg) contained the CPA oxime. The reaction product (4.2 mg, 10.3 μmol) was dissolved in 0.3 mL of DMF. To this solution, 4.2 mg of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, dissolved in 0.3 mL of double-distilled H_2O was added with stirring. The final pH of this mixture was 6.4. Activation was allowed to proceed with stirring for 15 min at RT. BSA (20 mg, 0.3 μmol), dissolved in 2 mL of H_2O/dimethylformamide (1:1), was then added to the activated oxime. Then 4.2 mg of 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide in 0.3 mL of double-distilled H_2O was added. The pH was adjusted to 6.4, and the mixture was stirred for 16 h at RT, followed by dialysis against 2 L of 10% dimethylformamide for 1 day, 5 L of deionized water for 2 days, and 5 L of glass-distilled water for a further day at 4 °C. The product was lyophilized and yielded 19.3 mg of the fluffy white CPA–BSA conjugate. This was redissolved in PBS, pH 7.4 (1 mg/mL), and stored in aliquots at −18 °C.

Conjugation of alkaline phosphatase (Boehringer Mannheim, 3000 units/mg of protein) to CPA or CPA-*O*-carboxymethyloxime was carried out in the same way as described above. The enzyme activity of the conjugates was determined spectrophotometrically with *p*-nitrophenyl phosphate as substrate [ϵ_{405} = 14 737 L/(mol cm)].

Immunizations and Antibody Preparation. Outbred rabbits 12–14 weeks old were used for the immunizations. The immunization protocol and blood sampling techniques have been described (Weiler, 1986). Antibodies were purified from the sera by the rivanol–ammonium sulfate precipitation technique described by Hurn and Chantler (1980) and stored in lyophilized form at −18 °C.

Enzyme-Linked Immunosorbent Assay (ELISA). Polystyrene plates (96-well, F-form, ELISA grade, Greiner or Nunc) were coated with 0.2 mL of the antibody solution (0.01 mg/mL in 50 mM $NaHCO_3$, pH 9.6) for at least 72 h at 4 °C. After the plates were decanted and rinsed with deionized water, all wells were incubated with 0.2 mL of 0.1% w/v gelatin and 0.05% (v/v) Tween 20 in Tris-buffered saline (50 mM Tris, 0.15 M NaCl, 1 mM $MgCl_2$, pH 7.8, TBS). Blocking was carried out for 1 h at RT. The plates were decanted and washed with deionized water and were now ready for use.

For the assay, 0.05 mL of TBS was added to each well followed by (i) 0.1 mL of TBS (to wells designed to determine maximum tracer binding, B_0), (ii) 0.1 mL of CPA standard dilution (to wells designed to determine the standard curve) [unspecific binding (UB) was determined by adding excess (1 nmol) CPA], or (iii) 0.1 mL of diluted sample. All standards, blanks, and samples were pipetted in triplicate. The plates were incubated for 15 min at 37 °C. Then 0.05 mL of alkaline phosphatase labeled CPA (dilution 1:1000, 49 pkat of enzyme activity) was added, and the plates were incubated for a further 2 h at 37 °C. The plates were then decanted and rinsed with deionized water. To determine the activity of the enzyme conjugate which was bound to the polystyrene-adsorbed antibodies, 0.2 mL of *p*-nitrophenyl phosphate, dissolved in diethanolamine buffer (1 M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8) at a concentration of 1 mg/mL was added to each well. After incubation for 1 h at 37 °C, the reaction was stopped with 0.05 mL of 5 N KOH, and the absorption of the samples was differentially read at 405–620 nm.

Extraction and Preparation of Samples for Immunoassay. Samples from agar surface cultures of fungal strains were punched with a sterile cork borer (8-mm diameter). Usually, five sample disks were collected from areas of the plates well covered with 4-week-old mycelium including the agar. Each disk represents ca. 0.17 mL of agar. The sample disks obtained from each plate were extracted separately by shaking in 1 mL of methanol/disk for 16 h at 4 °C. An aliquot of the methanolic extract (0.1 mL) was removed and diluted 10-fold with TBS. Aliquots of 0.1 mL of this dilution, representing 1/100th of the initial extract, were taken for analysis by ELISA.

For immunohistogram experiments, aliquots (0.1 mL) of the original methanolic extracts obtained as described above were applied to oxalate-impregnated silica gel plates. These were produced by spraying Merck silica gel 60 F_{254}, 0.2 mm, precoated plastic sheets (200 × 200 mm) with 1% (w/v) oxalic acid followed by heating for 30 min at 100 °C. The plates were developed with ethylacetate–2-propanol–ammonia (20:15:10 v/v) as the mobile phase (Betina, 1985). The dried chromatograms were cut into strips. Each strip was eluted with 1 mL of methanol overnight at 4 °C. An aliquot (0.1 mL) of each eluate was mixed with 0.9 mL of TBS, and 0.1-mL aliquots of the dilute samples were subjected to analysis by ELISA.

To determine the recovery of CPA from the agar, sterile agar plates were artificially contaminated with CPA at several concentrations. The toxin was first dissolved in methanol and then diluted with distilled water and filter sterilized. The appropriately diluted solutions were added to the medium while still fluid to give final concentrations of CPA from 0.04 to 0.4 μg of toxin/mL. The medium was then poured into the Petri dishes under sterile conditions and allowed to solidify, and samples for extraction were taken as described above.

RESULTS AND DISCUSSION

Generation and Properties of Antisera against CPA. Because of its low molecular weight, CPA is not immunogenic per se and thus has to be conjugated to protein prior to immunization. The site of coupling usually has a large influence on the properties of the resulting antibodies. Therefore, two types of immunogens were synthesized and used for immunizing rabbits according to an established protocol (Weiler, 1986). It was found that the CPA-*O*-carboxymethyloxime–BSA conjugate elicited a strong immune response in all three rabbits immunized. These antisera recognized both types of CPA–enzyme conjugates used in this study (see Materials and Methods), but displacement with excess CPA in both cases was slight (3–20%).

In contrast to this, the CPA–BSA conjugate prepared by the Mannich reaction elicited a weaker, though consistent, overall immune response. All of the animals immunized produced suitable titers of anti-CPA antibodies from the first bleed and maintained a constant titer upon prolonged immunization. These sera recognized only the homologous tracer, i.e., CPA coupled to the enzyme by the Mannich reaction. Displacement of the tracer from the antibodies by excessive CPA was complete. Therefore, this system was further characterized. From the collection of sera, one subset (from animal 527) was selected. After an initial screen, suitable serum fractions from different bleeds were pooled to yield the immunoglobulin fraction by standard techniques (Hurn and Chantler, 1980). This pool was used in all subsequent experiments. Optimum coating of the assay plates was achieved using 10 μg of the lyophilized antibody preparation/mL of 50 mM carbonate buffer, pH 9.6, and a coating period of at least 72 h at 4 °C. It was found that prolongued coating resulted in considerably less within-plate variation of antibody adsorption than usual coating times which vary from 1 to 24 h. The CPA–enzyme conjugate was diluted such that under standard assay conditions [ΔOD (405–620 nm), B_0] − [ΔOD (405–620 nm), UB] = 0.6. This was achieved at a final tracer dilution of 1:1000 (49 pkat of enzyme

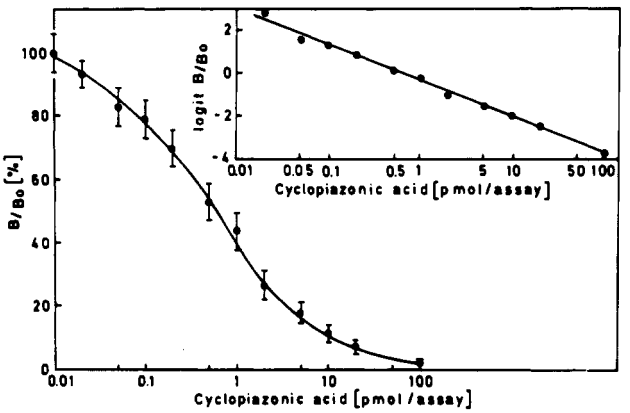

Figure 1. B/B_0 plot and logit–log plot (insert) of the standard curve for the competitive ELISA of cyclopiazonic acid (means and standard deviations calculated from 30 separate assays run on different days).

Table I. Characteristic Parameters for the Competitive ELISA of Cyclopiazonic Acid

parameter	value
coating immunoglobulin concentration, μg/mL	10
amount of CPA–enzyme tracer per well, pkat	49
measuring range, pmol of CPA/assay	0.1–100
midrange, (B/B_0 = 50%), pmol/assay	0.6
unspecific binding, %	2
intraassay variability, %CV[a] (n = 5)	3.7
interassay variability, %CV[a]	
within day (n = 5)	10.2
between days (n = 30)	14.0

[a] Averaged for all standards throughout measuring range.

activity per well). The timing of all assay steps was based on detailed CPA and tracer binding kinetics (not shown) which were found to follow usual time courses. TBS buffer at pH 7.8 gave optimum results as incubation buffer. Tris alone was less efficient. The pH optimum of the reaction was very pronounced, and binding sharply decreased below pH 7 and above pH 9.

Assay Sensitivity and General Characteristics. The CPA standard curve obtained for the optimized assay is shown in Figure 1, while in Table I the characteristic assay parameters are listed. The linear portion of the logit–log plot (Figure 1, insert) extends from 0.02 to 100 pmol of CPA/assay (ca. 10 pg–30 ng of CPA). For reading of sample values, only the range from 0.1 to 5 pmol (30 pg–2 ng) of CPA (B/B_0 from 80 to 20%) was used to ensure maximum precision. Unspecific binding was acceptably low (2%), and the statistical data (cf. Table I) demonstrate the high reproducibility of the standard curve.

Assay Specificity. Several structurally or functionally related compounds (1–7, Figure 2) were assayed for their cross-reaction in the assay, among them the biosynthetic precursor of CPA, tryptophan (5), tenuazonic acid (2), an *Alternaria* mycotoxin biosynthetically related to CPA (Stickings and Townsend, 1961), the tremorgenic mycotoxins fumitremorgin B (3) and penitrem A (7), and two further indole metabolites [ergocristin (4) and indole-3-acetic acid (6)]. None of these structures showed any detectable cross-reaction (tested up to 50 nmol/assay). Compared to the midrange value (B/B_0 = 50%) of 0.6 pmol/assay, this means that any potential cross-reaction of these metabolites will be much lower than 0.0012%. The lacking cross-reactivity of tenuazonic acid (2), tryptophan (5), and simple indoles such as indole-3-acetic acid (6) shows that neither the acetoacetate-derived portion of CPA nor the indole nucleus alone is sufficient for antibody binding. The antibodies are thus highly selective for CPA.

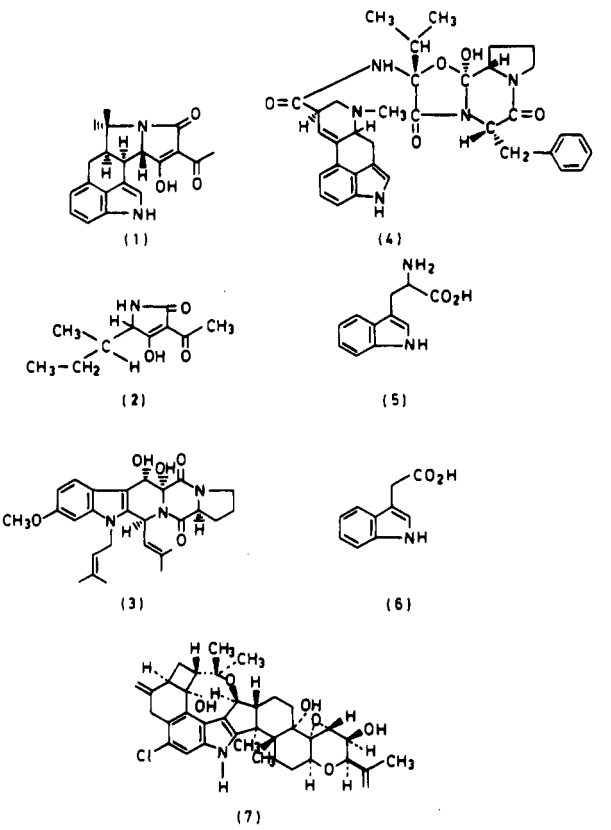

Figure 2. Structures of cyclopiazonic acid (1) and related compounds assayed for cross-reactions (2–7).

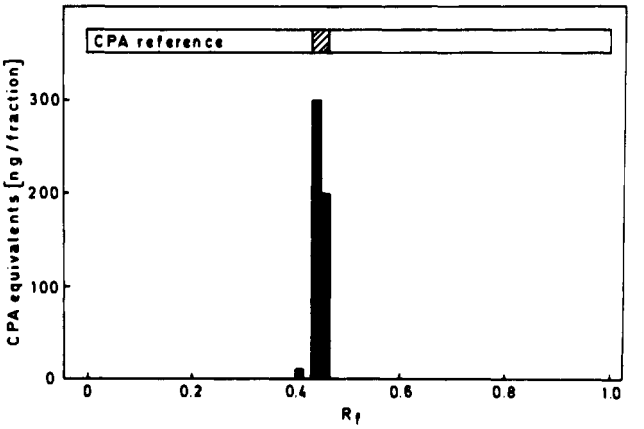

Figure 3. Distribution of immunoreactive material on a thin-layer chromatogram of a *P. camemberti* Sp 438 extract. For all details, see Materials and Methods.

Assay selectivity was further tested by the following experiment: a crude methanolic extract from surface cultures of *P. camemberti* Sp 438, an efficient CPA producer, was separated by TLC, and all zones of the chromatogram were eluted and analyzed for immunoreactivity (Figure 3). Only a single immunoreactive zone at the R_f of CPA was detected. A similar analysis was carried out for *A. flavus* ATCC 36182, with identical results (not shown). These results show that intermediates in the biosynthesis of CPA or structurally related metabolites either are not recovered by our extraction procedure, occur at levels too low to be detectable, or are not cross-reacting with our antibody. The assay reported here thus allows the selective analysis of CPA in crude extracts from fungal agar surface cultures.

Production of CPA by Fungal Cultures. A number of fungal reference strains, among them known producers of CPA and taxonomically unrelated species not suspected

Table II. Distribution of Cyclopiazonic Acid in Surface Agar Cultures of *A. flavus* ATCC 36182 and *P. camemberti* Sp 438

sample assayed	CPA, μg/mL of agar	
	A. flavus[a]	*P. camemberti*[b]
control agar	0	0
mycelium just visible	37	nd[c]
thin, growing mycelium	138	nd
agar surrounding well-established, young mycelium	158	0.4
well-established, young mycelium	333	1.4
dense, old mycelium	501	2.0

[a] Age of culture 46 days. [b] Age of culture 21 days. [c] nd, could not be determined because of compact growth pattern of fungus.

Table III. Recovery of Cyclopiazonic Acid from Agar (Medium MEN)

CPA added, μg/mL	CPA recovered,[a] μg/mL	recovery, %
0.042	0.034 ± 0.059	81
0.105	0.12 ± 0.017	114
0.21	0.23 ± 0.015	110
0.42	0.38 ± 0.008	90

[a] Mean ±SD from a triplicate analysis.

to contain the toxin as well as several isolates from commercial white or blue mold fermented cheeses, were analyzed in a pilot experiment to demonstrate the usefulness of the assay for rapid identification of CPA producers.

Under the growth conditions specified under Materials and Methods, all known CPA producers tested formed readily detectable, and some formed large, quantities of the toxin during surface culture on agar. A typical set of data is shown in Table II. It can be seen that (a) the culture medium alone gives zero background, (b) CPA can be detected as soon as growing mycelium becomes visible on the plates, (c) the toxin is apparently secreted into the agar because it can be extracted from the diffusion zone surrounding the mycelium, and (d) the highest levels of CPA can be extracted from plates well covered with older mycelium. CPA levels strongly increase with the age of the mycelium, suggesting a continuous production of the toxin during the growth and even stationary phase of culture.

The recovery of low levels (0.04–0.4 μg/mL of agar) of CPA from the agar under the extraction conditions chosen was, on the average, 99%. A typical recovery experiment is shown in Table III. The limit of detection of CPA standard from such agar samples under the conditions of the experiment is 0.02 μg/mL of agar. The detection limit for CPA from mycelium-covered plates could be estimated from the data obtained for the non-CPA-producing reference strains analyzed (Table IV) and was found to be 0.2 μg of CPA/mL of agar. The data show that while the actual amount of CPA extracted from a surface culture may vary considerably with the age of the culture, the technique allows detection, with high recovery, of even traces of CPA formed early in fungal growth. This allows the unambiguous identification of even weak producers of the toxin.

Table IV compares the production of CPA, as measured by ELISA, of a range of fungal reference strains as well as blue-mold and white-mold isolates from cheese samples. The CPA production of the reference strains in each case was as expected with known strong CPA producers giving high readings in our system and nonproducers giving zero to trace values. By comparison to the reference strains, all of the blue-mold isolates tested were negative while all of the white-mold isolates were significantly positive. This

Table IV. Cyclopiazonic Acid Production by Agar Surface Cultures of Fungal Strains

strain	major known mycotoxin	CPA,[a] μg/mL of agar
A. Non-CPA-Producing Reference Strains		
Alternaria alternata	tenuazonic acid	0.0082 ● 0.0076
Alt. tenuissima	tenuazonic acid	0.023 ± 0.023
A. caespitosus	verruculogen	0.12 ± 0.18
A. fumigatus	verruculogen	0.14 ± 0.19
P. ochrachloron	verruculogen	0.05 ± 0.09
P. paxilli	verruculogen	0.15 ± 0.19
P. simplicissimum	verruculogen	0.09 ± 0.17
P. verruculosum	verruculogen	0.09 ± 0.17
B. CPA-Producing Reference Strains		
A. flavus	CPA, aflatoxins	157 ± 148
P. camemberti		
Sp 438	CPA	82 ± 53
SP 1038	CPA	24.4 ± 9.6
P. commune	CPA	24.6 ± 15.0
P. puberulum	CPA	33.6 ± 18.5
P. verruculosum		
var. album	CPA	8.0 ● 5.8
var. cyclopium	CPA	6.8 ± 3.1
C. Blue-Mold Isolates		
SK 1		0.005 ± 0.01
SK 2		0.13 ± 0.25
SK 3		0.008 ● 0.02
SK 4		0.008 ± 0.002
SK 5		0.008 ● 0.002
SK 6		0.016 ± 0.03
SK 7		0.028 ± 0.12
SK 8		0.010 ± 0.12
D. White-Mold Isolates		
WSK 1		2.4 ± 2.0
WSK 2		5.9 ± 3.1
WSK 4		22.0 ± 5.2
WSK 5		15.5 ± 4.5
WSK 6		17.2 ± 5.4
WSK 8		3.4 ± 2.0

[a] Mean ±SD of $n \geq 3$ independent analyses.

confirmed the conclusion of earlier studies that CPA production is a common feature of industrial white-mold strains [e.g., Le Bars (1979), Still et al. (1978), and Geisen et al. (1990)].

ABBREVIATIONS USED

B_0, maximum binding; BSA, bovine serum albumin; CPA, cyclopiazonic acid; ELISA, enzyme-linked immunosorbent assay; HPLC, high-pressure liquid chromatography; PBS, phosphate-buffered saline; RT, room temperature; TBS, Tris-buffered saline; TLC, thin-layer chromatography; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; UB, unspecific binding.

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