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Cyclopiazonic Acid Production by *Penicillium camemberti* Thom and Natural Occurrence of This Mycotoxin in Cheese

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Every *Penicillium camemberti* strain freshly isolated from 20 commercial cheese brands produced cyclopiazonic acid in two culture media at 25, 13, and 4°C; the toxin yield was greatly dependent on the strain and environmental parameters (medium, temperature, and incubation time). The toxigenic ability appeared as a log-normal distribution. This mycotoxin was found in the crust (0.05 to 0.1 μg/g in three samples, 0.1 to 0.2 μg/g in five samples, and 0.4, 1, and 1.5 μg/g in three other samples) but not in the inner part. When its acute toxicity is considered, doses eventually ingested by consumers are very low (lower than 4 μg). Means for prevention are discussed. A highly toxigenic strength and rate appear to be necessary features leading to natural contamination in cheeses. The distribution of toxigenic ability makes possible without delay a choice of weakly toxic strains.

Cyclopiazonic acid, isolated from *Penicillium cyclopium* Westling (4), is toxic to rats per os (12); we found this toxin in laboratory cultures of 25% of *P. cyclopium* strains from feedstuffs. Other fungal species are also known to produce cyclopiazonic acid: *Aspergillus versicolor* (10), *Aspergillus oryzae* (11), and *Aspergillus flavus* (7). Recently, Still et al. (14) described cyclopiazonic acid in *Penicillium camemberti* cultures and in cheeses stored at 25°C, an unusual condition. Several types of cheeses (Brie, Camembert, Carré de l'est, etc.) are fermented by *Penicillium caseicolum* Bainier (9), which is now regarded as a synonym of *P. camemberti* Thom (13).

In relation to the hygienic hazard and the economic incidence of cyclopiazonic acid contamination, the purpose of this study was to examine toxin bioproduction by freshly isolated strains of *P. camemberti* from commercial cheeses and to investigate the possible occurrence of this toxin in these foods.

MATERIALS AND METHODS

Twenty *P. camemberti* strains were isolated from different commercial brands of Camembert cheese, purchased in a local supermarket, on a medium containing malt (6%), NaCl (6%), agar (2%), penicillin (30 IU/ml), streptomycin (30 μg/ml), and novobiocin (25 μg/ml).

Culture conditions. Each strain was cultured on a semisynthetic medium (medium A) with a high level of nitrogen and carbohydrates (Czapek solution plus 16% sucrose and 2% yeast exfract) and a synthetic one (medium B) (10) which was poor in nitrogen and contained the following (grams per liter): mannitol, 30; glucose, 10; succinic acid, 10; KH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.3; NH_4OH to pH 5.6. Fungi were grown in 30 ml of medium in 100-ml Erlenmeyer flasks at 25°C (optimal temperature for cyclopiazonic acid bioproduction) (14) for 25 days and at 13°C (ripening temperature for cheeses) and 4°C (refrigerator temperature) for 33 days.

Cyclopiazonic acid analysis. The development of cyclopiazonic acid concentration was examined in culture filtrates, and the yield in mycelia was measured at the end of each incubation period.

Thin-layer chromatographic (TLC) adsorbents were as follows: Silica Gel 60, 0.25-mm thickness, on glass plates (P), and Silica Gel 60, 0.25-mm thickness, on glass plates with fluorescent indicator (F), both impregnated with oxalic acid (O) or tartaric acid (T). Developer solvents were as follows: (solvent I) ethyl acetate-2-propanol-NH_4OH (20:15:10); (solvent II) $CHCl_3$-isobutylmethylketone (4:1); (solvent III) toluene-ethyl acetate-formic acid (5:4:1); (solvent IV) $CHCl_3$-acetone (95:5). Cyclopiazonic acid was visualized as a violet spot, in ordinary light, after spraying with Ehrlich reagent (10 g of 4-dimethylaminobenzaldehyde in 100 ml of HCl, extemporaneously diluted with 4 volumes of acetone).

Extraction procedures were as follows. A 1-ml portion of culture filtrate, adjusted to pH 3 with aqueous HCl (50:50), was extracted twice with 4 ml of $CHCl_3$; after drying and evaporation, 10 μl of the extract, dissolved in 0.1, 0.25, or 1 ml of $CHCl_3$, was spotted on TLC plates (P) and developed with solvent I. At the end of the incubation period, mycelial mats were extracted by soaking and agitation in an azeotropic $CHCl_3$-MeOH mixture; after drying and evaporation, extracts were dissolved in $CHCl_3$ for TLC; defatting was accomplished on TLC plates (P) and P-O by a predevelopment with toluene. Dried plates were then developed with solvents I and II, respectively; the quantitation limit was 0.5 μg/g. Mycelial mats, washed

twice with water, were weighed after drying at 105°C for 18 h. The crust of 20 cheeses (15 among them corresponding to isolated strains) and the inner part of 4 of them were twice extracted with azeotropic $CHCl_3$-MeOH. The filtered and evaporated extracts were dissolved in the following mixture: 20 ml of acetone, 60 ml of water, and 20 ml of lead acetate solution (200 g of lead acetate·$3H_2O$ plus 2 ml of acetic acid and in 1 liter of water). A 20-ml amount of a saturated Na_2SO_4 solution was poured on the extract; 5 g of Celite was added to the suspension, which was filtered on fritted glass through a 1-cm Celite pad. After defatting twice with hexane (80 ml) and acidification to pH 3 with aqueous HCl (50:50), the lower phase was extracted with $CHCl_3$ (60 and then 30 ml); this extract was centrifuged (3,000 rpm), dried with Na_2SO_4, evaporated, and dissolved in a minimal volume of 100 μl of $CHCl_3$. The TLC procedure was the same as that for mycelial extracts. The lowest detectable level was 0.02 μg of cyclopiazonic acid per g.

Quantitation was accomplished on TLC plates by comparison with a standard range of concentrations of cyclopiazonic acid (10, 20, 30, and 40 ng in 10-μl spots) in $CHCl_3$, prepared from a spectrophotometrically measured MeOH solution (log $\epsilon = 4.31$ at λ_{max} 284 nm; molecular weight = 336 [4]).

Cyclopiazonic acid identification from mycelia and filtrates was confirmed, in comparison with a standard (pure cyclopiazonic acid isolated from *P. cyclopium* cultures and compared with a sample given by P. S. Steyn), in the following ways: (i) different TLC techniques—P/solvent I, $R_f = 0.37$; P-O/solvent II, $R_f = 0.71$; P-O/solvent III, $R_f = 0.76$; P/solvent IV, $R_f = 0$; (ii) spectrophotometric characteristics of the isolated metabolite—ultraviolet (MeOH) 225, 253, 275 (sh), 284, 292 (sh) nm; (iii) melting point (decomposition)—243 to 246°C, crystallized from MeOH; (iv) desorption and fragmentation mass spectrometry—336 M^+, 196, 182, 181, 155, 154. From commercial cheeses, confirmation was realized by TLC procedures and, for one sample extract, by mass spectrometry, after a second purification step described below.

Pure cyclopiazonic acid was obtained from $CHCl_3$ mycelia and filtrate extracts by partition against a $NaHCO_3$-Na_2CO_3 solution (0.5 M, pH 9); after acidification of the aqueous phase (pH 3) with aqueous HCl (50:50), toxin was drawn off with a new $CHCl_3$ phase and submitted to preparative TLC (F-T)/solvent II. The cyclopiazonic acid band was collected and eluted with $CHCl_3$; tartaric acid was removed by washing three times with water. Cyclopiazonic acid was twice crystallized from MeOH.

RESULTS

Every strain grew under the different culture conditions. However, the lag phase lasted for 2 weeks at 4°C. The higher mycelial growth in medium A, rich in organic matter, was all the more considerable as the temperature was low (Table 1). Cyclopiazonic acid was found in every strain and under every culture condition, but its yield was greatly dependent on the strain and environmental parameters (medium, temperature, and incubation time).

The percentage of producing strains increased with time (Fig. 1) and reached 100% on day 22 at 25°C and on day 33 at 13 and 4°C. The toxigenic strength was quite variable, as shown by yield dispersions (Fig. 1). The toxigenic ability, expressed as the mycelial toxin concentration, appeared as a log-normal distribution (Fig. 2); 75% of the strains contained less than 400 μg/g; the highest values were 1,785 and 2,080 μg/g.

Mean concentrations in filtrates were similar in the two media at 25°C and higher in medium A at 13°C (Fig. 1). In mycelia, the mean toxin yield per culture flask was slightly lower in medium B, but concentrations were much more important than in medium A (Table 1).

In filtrates, at 25°C the mean concentration was 20 to 30 times higher than at 13°C on days

TABLE 1. *Mean values of mycelial dry weight and cyclopiazonic acid quantities per flask and concentrations in mycelium of 20 P. camemberti strains cultured on media A and B*

Temp (°C)	Incubation period (days)	Culture medium	Mycelial dry wt (mg)	Cyclopiazonic acid	
				Quantity (μg)	Concn (μg/g)
4	33	A	463	0.7	1.6
		B	18	0.5	30.7
13	33	A	846	57.3	67.8
		B	81	39.2	484
25	25	A	480	130	272
		B	101	83.6	828

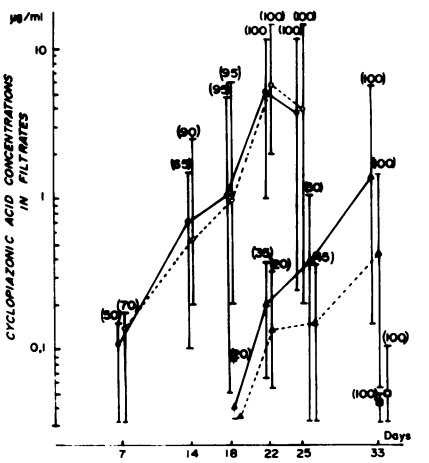

FIG. 1. *Development of cyclopiazonic acid concentrations (mean value and dispersion) in culture filtrates of 20 P. camemberti strains according to time and temperature. The percentage of producing strains is given within parentheses. Medium A: at 25°C (●); at 13°C (▲); at 4°C (■). Medium B: at 25°C (○); at 13°C (△); at 4°C (□).*

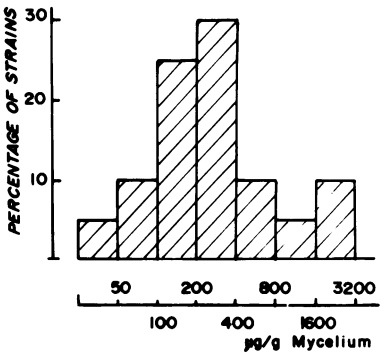

FIG. 2. *Toxigenic strength distribution of P. camemberti strains: cyclopiazonic acid concentration in mycelium (culture medium B).*

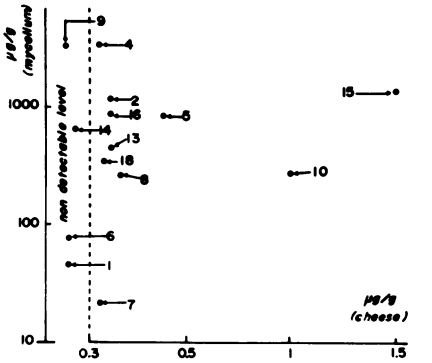

FIG. 3. *Comparison of cyclopiazonic acid concentrations in mycelia of several P. camemberti strains (culture medium B, 25°C) and in corresponding cheese crusts.*

18, 22, and 25; at 4°C, toxin was detectable (0.04 to 0.05 μg/ml) only after 1 month (Fig. 1). The mean concentration increased from 0.1 to 6 μg/ml between days 7 and 22 at 25°C and from 0.04 to 1.5 μg/ml between days 18 and 33 at 13°C. In mycelia, toxin amounts and particularly concentrations increased with the temperature; therefore, this exerts a direct effect on cyclopiazonic acid biogenesis (Table 1).

Multiple interactions were apparent between the strain factor and the environmental parameters: the better medium for toxinogenesis was not the same for every strain; the toxin production rate was not uniform, and the earliest producing strains were not always the highest producers; the effect of temperature on toxinogenesis was variable (e.g., for the same production at 13°C, strain no. 11 contained, at 25°C, 50 times more toxin than did strain no. 7 [1,000 versus 20 μg/g]).

Cyclopiazonic acid was present in 11 out of 20 cheese crusts in the following concentrations: 0.05 to 0.1 μg/g in three samples, 0.1 to 0.2 μg/g in five samples, and 0.4, 1, and 1.5 μg/g in the three other samples. It was not detected in the inner part of the four cheeses with the highest cyclopiazonic acid concentrations in the crust.

A comparison of concentrations in the mycelia and in the crust of the corresponding cheese (Fig. 3) showed the following: little or no yield in samples fermented by a weak producer strain; a noticeable concentration in cheeses fermented by good toxin producers (no. 5, 10, and 15); a nondetectable level in some samples fermented by a highly toxigenic strain (no. 14 and 9). Moreover, every sample with a cyclopiazonic acid content greater than 0.2 μg/g was fermented by a strain showing a comparatively high toxin production rate. Therefore, the "toxigenic" character is a necessary, but not a sufficient, condition to lead to natural cyclopiazonic acid contamination in such cheeses.

DISCUSSION

The above results demonstrate the presence of cyclopiazonic acid as a natural contaminant in cheeses purchased in a supermarket. According to Still et al. (14), toxin bioproduction occurs in these foods only at unusually high storage temperatures. In the present case, the frequency of this toxin in different cheese brands could be due to a defect in the course of marketing channels; however, we observed that even at 4°C, cyclopiazonic acid was produced in culture media. Luck et al. (6) did not find this toxin in the inner part of cheese, but the sensitivity of the technique was not specified. In cyclopiazonic acid-contaminated cheeses, the toxin remains in the mold layer.

Cyclopiazonic acid doses eventually ingested by consumers are very low compared with the 50% lethal dose in rats (36 mg/kg, per os [12]): 3 or 4 μg in a portion (one-eighth of a Camembert cheese) of the most contaminated sample. However, the different aspects of cyclopiazonic acid toxicity are not known. Several authors have examined the possible toxicity of *P. camemberti*. One culture extract was carcinogenic by oral and subcutaneous routes (3). On the contrary, in different biological tests, culture extracts were slightly or not toxic (5, 8). Long-term feeding experiments with rainbow trout (2) and rats, using *P. camemberti* cultures and cheese, as well as subcutaneous application of the mycelia, did not give any indication of a detrimental or carcinogenic effect (1).

Nevertheless, in the improvement of product quality, no aspect can be neglected, particularly from the viewpoint of human health. Taking

into account the present data, means of prevention, compatible with manufacturing technology, must address themselves to the choice of strains and the observance of correct storage conditions. A highly toxigenic strength and rate appear to be necessary features leading to natural contamination in cheeses; the distribution of the toxigenic ability, similarly observed elsewhere (J. Le Bars, Ann. Rech. Vet., in press) makes possible without delay a choice of weakly toxic strains.

Given multiple interactions between strains and culture conditions, such a selection must be realized with the natural substrate under normal fermentation and storage conditions. The main characteristics to examine for a strain would be the following, in this order: no (or weak) toxinogenesis; slight effect of temperature increase to prevent possible failures in marketing channels and some consumer practices; low toxinogenesis rate.

Meanwhile, additional knowledge of cyclopiazonic acid toxicity and its natural occurrence is urgently needed.

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