Production of the Hepatotoxic Chlorine-Containing Peptide by *Penicillium islandicum* Sopp

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Received for publication 7 May 1973

Twenty-six strains of *Penicillium islandicum* Sopp were tested for production of a chlorine-containing peptide (Cl-peptide), which is a hepatotoxin. Highest levels of the mycotoxin were produced in a modification of Wickerham medium. The mycotoxin was isolated by adsorption on charcoal and, after washing the charcoal with acetone-water (1:1), was eluted into n-butanol. Further purification was accomplished by gel filtration. Maximum yields were 10 to 20 mg of toxin per liter of culture filtrate.

*Penicillium islandicum* Sopp, one of the causes of yellow rice toxicosis, produces two hepatotoxins. One hepatotoxin is an anthraquinoid pigment, luteoskyrin, from the fungal mat. The other hepatotoxin is a chlorine-containing cyclic peptide (Cl-peptide) from the culture filtrate (6).

Long-term feeding experiments revealed that these two mycotoxins induced tumorous changes in the livers of mice (5). Since large amounts of the mycotoxins were needed for toxicological tests, the authors determined the optimal conditions for the production of luteoskyrin and established a procedure for its isolation (4).

As to the Cl-peptide mycotoxin, the content in the culture broth was very low, and the toxin-producing capability diminished during storage of the fungi. These factors hindered the development of a procedure for the production of Cl-peptide, and consequently toxicological studies were abandoned about 10 years ago.

Recently, the authors renewed their efforts to obtain a high yield of Cl-peptide. A photometric method for the estimation of minute quantities of Cl-peptide and a procedure for purification of the toxin by gel filtration were reported (1). In the present paper, improved methods for cultivation of the fungus and extraction of Cl-peptide are described. In these methods, *P. islandicum* Sopp WF-38-12 was cultured on modified Wickerham medium, and crystalline Cl-peptide was isolated from the culture filtrate by a combination of charcoal adsorption, n-butanol elution, and gel filtration.

MATERIALS AND METHODS

Strains of *P. islandicum* Sopp were generously supplied by H. Tsunoda (Food Research Institute), H. Kurata (Institute of National Hygienic Sciences), and M. Yamazaki (Chiba University). The fungal strains were inoculated and stored on malt-dextrose-agar slants or rice grains at 4 C.

Five hundred-milliliter Erlenmeyer flasks or petri dishes (21 cm diameter), each containing 200 ml of a liquid medium, were sterilized and inoculated with 1 ml of the conidial suspension. After stationary cultivation at 25 C for 14 days, the culture broth was filtered through filter paper and the fungal mat was dried overnight at 60 to 70 C. The media examined for ability to support production of Cl-peptide were: (i) Czapek medium; (ii) Czapek medium plus 1% peptone, yeast extract, malt extract, or corn steep liquor; (iii) Wickerham medium (2) modified by omitting glucose and increasing the sucrose content to 4%; (iv) malt dextrose medium; (v) Sabouraud medium; (vi) Mayer medium.

For toxicity tests, culture filtrates or fractions dissolved in water were administered intraperitoneally (i.p.) to male mice and lethality was determined 7 days after administration.

Chemical determination and gel filtration of Cl-peptide were carried out by the methods previously reported (1). Luteoskyrin, after being extracted from the fungal mat, was detected on thin-layer chromatography (TLC) plates coated with 0.1 M oxalic acid-treated Kieselgel G; the solvents were acetone-n-hexane-water (4:2:1 vol/vol/vol).

1This paper is part of Yellow Rice Research Term Reports, Section 0.


RESULTS

Screening of the Cl-peptide-producing strains. Twenty-six strains of *P. islandicum* Sopp were cultured on Czapek medium, and the lethal toxicity of each culture filtrate was examined by i.p. administration to mice in doses of 0.25, 0.50, and 2.0 ml per 10 g of body weight; the 2.0-ml dose was a 10-fold concentrate of culture filtrate. Table 1 summarizes the toxicities of the culture filtrates as well as the yields of fungal mycelia and their relative contents of luteoskyrin. All of the fungal mycelia contained luteoskyrin, whereas the content of Cl-peptide in the culture filtrates differed markedly depending on the strain examined. The highest toxicity was observed with the strain WF-38-12, which killed all three test mice at an i.p. dose of 0.25 ml of filtrate/10 g of body weight. The livers of the dead mice were swollen to two to three times larger than those of the control animals and were dotted with small red spots; these properties are characteristic of injury by Cl-peptide.

| Table 1. Final pH, mycelial yields, and estimates of Cl-peptide and luteoskyrin levels produced by 26 strains of *Penicillium islandicum* |
|---|
| Strain | Final pH | Mycelial dry wt (g/liter) | Toxicity of culture broth (ml/10 g i.p.) | Luteoskyrin on TLC |
|---|---|---|---|---|
| JC | 4.65 | 7.5 | 3/3 | 0.5 | 0.25 | + |
| JC 1-2 | 4.70 | 7.2 | 3/3 | 0/3 | + |
| JC 43-1-9 | 4.48 | 7.3 | 1/3 | + |
| Akita | 4.50 | 7.3 | 3/3 | 0/3 | + |
| Akita-f | 4.85 | 6.6 | 3/3 | 0/3 | + |
| NEB | 5.00 | 7.7 | 3/3 | 0/3 | + |
| NEB 43-1-9 | 4.38 | 7.7 | 3/3 | 0/3 | + |
| E 43-1-9 | 4.55 | 6.7 | 2/3 | +++ |
| RF-11-2 | 4.52 | 7.2 | 2/3 | + |
| MR-202-6 | 4.48 | 6.5 | 2/3 | + |
| WF-38-1 | 4.70 | 6.4 | 3/3 | 0/3 | + |
| WF-38-12 | 4.70 | 6.4 | 3/3 | 3/3 | 3/3 | + |
| CU 0016 | 4.00 | 6.3 | 2/3 | + |
| CU 0017 | 4.20 | 5.3 | 0/3 | + |
| CU 0221 | 4.40 | 6.3 | 3/3 | + |
| CU 0222 | 3.80 | 5.2 | 1/3 | + |
| CU 0223 | 3.82 | 5.3 | 1/3 | +++ |
| CU 0224 | 3.90 | 5.0 | 0/3 | + |
| CU 0375 | 4.45 | 5.7 | 0/3 | + |
| CU 0522 | 4.10 | 5.9 | 2/3 | + |
| CU 0523 | 4.20 | 6.0 | 3/3 | 0/3 | + |
| CU 0555 | 4.00 | 6.1 | 3/3 | 0/3 | + |
| CU 0652 | 4.10 | 5.5 | 0/3 | + |
| CU 0653 | 4.00 | 5.5 | 0/3 | + |
| CU 0679 | 4.30 | 6.3 | 3/3 | 0/3 | + |
| CU 0722 | 4.15 | 5.9 | 0/3 | + |

* Concentrated 10 times when administered to mice.

Examination of extraction procedures. Eighteen liters of culture filtrate from strain WF-38-12 was mixed with 360 g of 1 N HCl-treated charcoal. After standing overnight at 4°C, the charcoal was removed from the culture filtrate and immersed in 5 liters of methanol for 14 h, followed by immersion in the same volume of *n*-butanol. The charcoal was removed by filtration and the yellow filtrate was evaporated to dryness; a brown material was obtained. Portions of the culture filtrate and methanol eluate were also evaporated to dryness. The dry matter content and lethal toxicity in mice of the culture filtrate and methanol and *n*-butanol eluates are shown in Table 2. Out of 18 g of solids/liter of the culture filtrate, the methanol elution extracted 370 mg with a lethal toxicity of 50 mg/10 g. On the other hand, the solids content of the subsequent *n*-butanol eluate decreased to one-fourth that of the methanol eluate, but the lethal toxicity increased to 0.5 mg/10 g; about 38% of the total lethal toxicity in the culture filtrate was recovered in the *n*-butanol extract.

As previously reported (4), charcoal is non-specific in its adsorption of organic metabolites from culture filtrates. Therefore, we attempted to remove the nonpeptide organic materials by washing the charcoal with various solvents and solvent mixtures before the toxin was eluted in *n*-butanol. We hoped thereby to increase the purity of toxin in the *n*-butanol extracts. The use of acetone-water (1:1) was superior to methanol, methanol-water (1:1), undiluted acetone, or acetone-water (8:2) (Table 3).

*P. islandicum* produces substantial quantities of organic acids and other acidic metabolic products; some of these compounds are recovered in the *n*-butanol fraction. To remove these acids, the culture filtrate was treated with...
charcoal, the charcoal was washed with acetone-water (1:1), and the toxin was eluted with n-butanol. The n-butanol fraction was then extracted with 1 M phosphate buffer (pH 7.0). The resulting upper n-butanol layer and lower buffer layers were separated and evaporated to dryness. The residues were then reextracted with hot methanol. The solids in the n-butanol extracts were equally distributed in the buffersoluble and n-butanol-soluble fractions (Table 4). The lethal toxicity, however, remained in the n-butanol fraction; this fraction is referred to as "crude toxin" in the present report.

**Examination of culture conditions.** A variety of media were first examined. Two liters of each of the nine media listed in Material and Methods were prepared, divided into samples of 250 ml each, dispensed into eight petri dishes (12 cm in diameter) for each medium, and inoculated with strain WF-38-12. After cultivation at 25 C for 14 days, each pooled culture filtrate (from the eight dishes) was mixed with 20 g of acid-treated charcoal. The charcoal was washed with 400 ml of acetone-water (1:1), followed by extraction with 400 ml of n-butanol. The final pH values, weights of the dried fungal mats and dry matter in the n-butanol extracts, and toxicities of the final preparations are summarized in Table 5. Czapek medium supplemented with peptone or yeast extract and Sabouraud medium supported rapid fungal growth, but the toxicity and the yield of the n-butanol extract were highest when the fungus was cultured on modified Wickerham medium (40 g of sucrose, 3 g of peptone, 2 g of yeast extract, 10 g of corn steep liquor, 2 g of NaNO₃, 1 g of K₂HPO₄, 0.5 g of MgSO₄·7H₂O, 0.5 g of KCl, and 0.01 g of FeSO₄ in 1,000 ml of water). On the other hand, TLC analyses revealed that the relative content of luteoskyrin in the fungal mat was highest when the fungus was cultured on unsupplemented Czapek medium.

To determine the time course of CI-peptide production, _P. islandicum_ Sopp WF-38-12 was cultured on modified Wickerham medium; at desired intervals, 2.4 liters of the culture was withdrawn to estimate the lethal toxicity of the culture filtrate, dry weight of the fungal mat, and content of the sugar added to the medium. Maximum growth of the fungus was attained after 8 days (Fig. 1). Carbohydrate utilization was coincident with fungal growth. The toxicity of the culture filtrate increased during the later stages of cultivation; maximum levels were reached by day 14 and persisted through day 20.

The amino acids which constitute CI-peptide (L-serine, DL-β-amino-β-phenyl propionic acid, L-α-amino-butyric acid, L-proline) were added to modified Wickerham medium at the initial or middle stages of fungal growth. Levels tested were 100 mg of each amino acid individually per liter and a mixture containing 100 mg of all four amino acids per liter. Amino acid supplementation did not increase the toxicity of the culture filtrate.

**Purification and isolation of CI-peptide.** To prepare a large amount of CI-peptide, _P.
**Table 5. Evaluation of culture media for Cl-peptide production**

| Medium               | Final pH | Mycelia* (g) | n-Butanol fraction* (mg) | Toxicity (mg/10 g) |
|----------------------|----------|--------------|--------------------------|--------------------|
| Czapek              | 4.2      | 6.25         | 88                       | 0/3                |
| Czapek-peptone       | 4.6      | 11.25        | 146                      | 3/3                |
| Czapek-yeast extract | 4.6      | 13.50        | 94                       | 3/3                |
| Czapek-malt extract  | 4.2      | 7.75         | 90                       | 3/3                |
| Czapek-corn steep liquor | 4.6    | 8.25         | 129                      | 3/3                |
| Modified Wickerham   | 4.4      | 10.50        | 244                      | 3/3                |
| Malt-dextrose        | 3.8      | 3.75         | 78                       | 2/3                |
| Sabouraud            | 4.2      | 11.25        | 144                      | 3/3                |
| Mayer                | 2.2      | 10.75        | 126                      | 3/3                |

*Based on 1 liter of culture broth.

**Fig. 1.** Time course of fungal growth, toxin formation, and changes in pH and sugar content in the culture medium. The fungus was cultured on modified Wickerham medium; 50 g of sucrose per liter was used in this experiment.

*P. islandicum* Sopp WF-38-12 was cultured in 100 petri dishes (21 cm in diameter), each containing 250 ml of modified Wickerham medium. Three grams of the crude toxin was obtained. The crude toxin was placed on a Sephadex LH-20 column (4.5 by 90 cm) and, as was previously reported (4), Cl-peptide was eluted with methanol-water (1:1) at a flow rate of 20 ml per fraction per 30 min. The elution of Cl-peptide was followed by measuring the optical density at 268 nm before and after ammonolysis with 2 N NH₄OH (1). Gel filtration of the crude peptide gave a sharp peak in fractions 76 to 86 and the ultraviolet spectrum of the maximum peak at no. 80 was identical to that of the ammonolized CI-peptide (1) (Fig. 2). Fractions 76 to 86 were pooled and the solvent was removed by evaporation. Crystalline needles appeared from a hot methanol solution of the residue. From the mp (255 C) and IR spectrum of the crystalline product, the crystals were identified as Cl-peptide. Two separate experiments yielded 100 and 200 mg of Cl-peptide from 20 liters of culture filtrate.

**DISCUSSION**

Among the 26 strains of *P. islandicum* tested, 20 strains were found to produce a toxic filtrate that killed mice in i.p. doses of 0.2 to 2.0 ml/10 g of body weight. On the other hand, all the strains were found to synthesize and accumulate hepatocarcinogenic luteoskyrin (Table 1). In addition to natural differences between strains in Cl-peptide-producing ability, there is a loss in capacity to synthesize the mycotoxin during subculture on a synthetic medium, such as Czapek agar. On the other hand, the fungi maintained the ability to produce Cl-peptide when rice grains were used as culture substrate for culture storage.

Supplementation of the Czapek medium with natural nutrients was found to accelerate the formation of Cl-peptide; this effect is one of the reasons the high levels of Cl-peptide production were attained on modified Wickerham medium,
Fig. 3. Procedure for isolation of Cl-peptide.

which consists of peptone, yeast extract, corn steep liquor, and other chemicals. The effect of organic supplements is important from the viewpoint of toxigenicity and food hygiene. In accordance with previous studies (4), the media supplements accelerated fungal growth and production of Cl-peptide, whereas the luteoskyrin contents of the fungal mycelia decreased. Furthermore, according to Tsunoda et al. (3), the toxicity of cereals infected with *P. islandicum* varies markedly, depending upon the cereal used. These results strongly suggest that the production of two hepatotoxins by *P. islandicum* is influenced by the contents of carbohydrates and nitrogenous nutrients in culture media.

We introduced the following two steps in the improved procedure for isolation of Cl-peptide: (i) washing with acetone-water of charcoal containing adsorbed Cl-peptide to remove nonpeptide materials before eluting the Cl-peptide; and (ii) using n-butanol to elute Cl-peptide (Table 2). In some cases, the Cl-peptide in crude toxin preparations could be crystallized without gel filtration. However, when the crude toxin preparations were chromatographed on Sephadex, the yields of crystalline Cl-peptide increased about three times. Therefore, gel filtration is considered to be an important step for preparation of Cl-peptide in large quantities. The improved procedure for isolation and production of Cl-peptide is schematically represented in Fig. 3.

ACKNOWLEDGMENTS

This investigation was aided in part by a grant for cancer research (1971) from the Ministry of Welfare.

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