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Penitrem and thomitrem formation by *Penicillium crustosum*

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Abstract

The levels of penitrems A, B, C, D, E, F, roquefortine C and thomitrem A and E recovered from extracts of 36 Norwegian, 2 American and one each of Japanese, German, South African, Danish and Fijian isolates of *Penicillum crustosum* Thom were quantitatively determined using high performance liquid chromatography-mass spectrometry (HPLC-MS). Forty-two of the 44 isolates of penitrem-producing isolates grown on rice, afforded levels of thomitrem A and E comparable to that of penitrem A. Thomitrems A and E were also found, but at lower levels, when cultures were grown on barley. No thomitrems were found when the isolates were grown on liquid media. The effects of time and temperature on mycotoxin formation were studied on rice over a 4 week period at 10, 15 and 25 °C, respectively. No mycotoxins could be detected after 1 week at 10 °C, but after 2 weeks at 10 °C levels were similar to those produced at 15 and 25 °C. Higher levels of thomitrems A and E were detected when media were maintained at lower pH. The possibility that thomitrems A and E might be derived by acid promoted conversion of penitrems A and E was explored in stability trials performed at pH 2, 3, 4, 5 and 7 in the presence and absence of media. Thomitrems were formed at pH 2, 3 and 4 but not at pH 5 and 7.

Key words: *Penicillium crustosum*, penitrems, thomitrem, foods, feeds

Introduction

We have recently reported the occurrence of two novel penitrem metabolites, thomitrems A and E, along with penitrems A, B, C, D, E and F from a single Norwegian *Pencillium crustosum* isolate 1590P2 grown on a solid rice medium [1]. The detection of thomitrems A and E, which unlike other known penitrem analogues, possess an 18(19)-double bond (Figure 1) is of interest in that so far the use of liquid cultures of *P. crustosum* have been reported to only afford roquefortine C, penitrems A–F [2-3] and derivatives such as penitremones [4], PC-M4, M5, M5′ and M6 [5–6]. Mycotoxins produced by *P. crustosum* have been reported to cause toxicity [7–9] in animals. Whether the thomitrems may contribute to *P. crustosum* toxicity has not been studied.

The unexpected occurrence of the thomitrems A and E in the extracts of the 1590P2 isolate [1], and our qualitative observation that some other Norwegian *P. crustosum* isolates also appeared to produce thomitrems, prompted us to quantatively examine the extracts of 36 Norwegian together with one Danish, one Fijian one Japanese, one Russian, one German, two American and one South African isolate of *P. crustosum* available in our laboratory in order to ascertain the generality, or otherwise, of our earlier findings. Additionally we also wanted to determine the effects of solid and liquid media, pH, temperature and incubation time on the formation of penitrems and thomitrems under the condition utilized in our solid culture medium experiments since temperature, water activity, medium and incubation time are known to be important factors affecting fungal growth and mycotoxin production [10].

Materials and methods

Penicillium strains

Thirty-six Norwegian isolates of *P. crustosum* from Norwegian food and food waste together with one

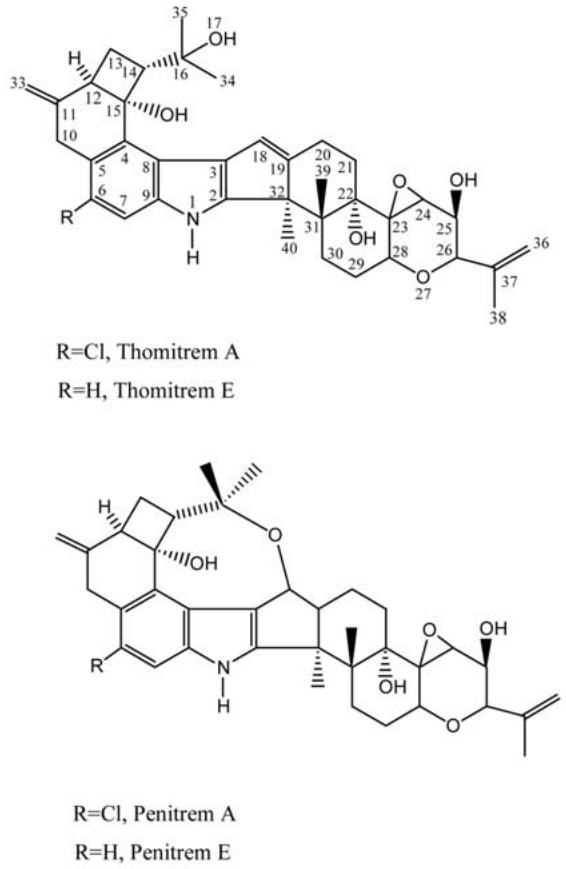

Figure 1. Chemical structure of thomitrem A, E, penitrem A and E.

Danish (IBN19484, Denmark Technical University, Copenhagen), one Fijian (CBS 313.48), one Japanese (CBS 340.59), one Russian (CBS 499.73), one German (CBS 483.75), two American (CBS 747.74 and CBS 548.77) and one South African isolate (PPR15859, ARC Plant Protection Research Institute, Pretoria) were studied.

Media

Rica and Barley: Twenty g of Uncle Ben's rice and 60 ml distilled water were added to 500 ml Erlenmeyer flasks and 20 g of unhusked barley and 12 ml distilled water were added to 250 ml Erlenmeyer flasks and sterilised by autoclaving for 15 min.

Liquid media: Twenty ml of Czapek Dox yeast extract broth (CDYE) [12] without agar were added to a 100 ml Erlenmeyer flasks. The media were sterilised by autoclaving for 15 min.

pH adjusted media: Ten ml of Czapek yeast (autolysate) extract broth (CYA) [11] without agar were added to a 50 ml Erlenmeyer flask and 0.1 M HCl was added dropwise to give an initial media pH of 2.5, 4, 5.5 and 7. The media were sterilised by autoclaving for 15 min.

All media were point inoculated from malt extract agar [11] cultures incubated for 6 days at 25 °C.

Incubation

All of the *P. crustosum* isolates were point inoculated and incubated on rice for 2 weeks at 20 °C. Four selected penitrem producing Norwegian isolates, 1172F10, 1574F8, 1590P5 and 1592P5 (National Veterinary Institute numbers) were point inoculated and incubated on barley and on CDYE for 2 weeks at 20 °C. A Norwegian strain, 1590P5, and the South African strain, PPRI6859, were point inoculated and incubated on rice for 1, 2, 3 and 4 weeks at 10, 15 and 25 °C. There were three replicates of each treatment. Another Norwegian strain, 1564F1, was point inoculated and incubated for 1 week at 25 °C on liquid CYA media in which the pH had initially been adjusted to 2.5, 4, 5.5 and 7.

PH stability trials

Trial 1: The stability of penitrem A in acid was investigated by treating an acetonitrile:water (2:1) solution containing 100 ng/ml penitrem A with 0.01 M HCl (pH 2). The acidic penitrem A solution was kept at room temperature (20 °C) and analysed by high performance liquid chromatographic-mass spectrometry (HPLC-MS) every other hour for 24 h.

Trail 2: A one week old CYA inoculate (10 ml) was diluted with 20 ml acetonitrile and placed in an ultra sonic bath for 10 min. The sample extract were filtered and the pH was adjusted to 3, 4 and 5 with 0.1 M HCl. The levels of penitrems and thomitrems were monitored at 12 h intervals for 72 h. The sample extracts was kept at room temperature (20 °C) between analysis.

Chemicals

Methanol, ethyl acetate, acetonitrile, hexane, ammonium acetate and formic acid were HPLC grade or p.a. grade obtained from Ratburn Chemicals (Walkerburn, U.K.). The *Penicillium* toxin standards roquefortine C, and penitrem A were obtained from Sigma (St. Louis, USA), thomitrems A and E were isolated at the National Veterinary Institute (Oslo, Norway), while penitrems B, C, D, E and F were gifts from

AgResearch Ruakura (Hamilton, New Zealand) and Imperial College of Science Technology and Medicine (London, UK).

Extraction and clean up

Rice and barley cultures

The extraction procedure was based on a method described by Rundberget and Wilkins [13]. Each of the rice or barley culture flasks were extracted with 100 ml acetonitrile:water (9:1) for 5 min on an Ultrathurax (Janke and Kunkel, Staufen, Germany). After defatting with 50 ml of hexane, the extract was filtered (Schleicher & Schuell 520 B $\frac{1}{2}$ folded filters, Dassel, Germany). This extract was filtered further with a spin-X centrifuge filter (Costar, Corning, USA) and then analysed on the described HPLC-MS system without further clean up.

CDYE cultures

Cultures were extracted by shaking with 50 ml ethyl acetate for 10 min. After filtration, 10 ml of the ethyl acetate fraction was evaporated to dryness at 60 °C under a gentle stream of nitrogen. The residue was redissolved in 900 ml acetonitrile and diluted with 100 ml water prior to HPLC-MS analyses.

pH adjusted CYA cultures

The CYA inoculate (10 ml) was diluted with 20 ml acetonitrile and placed in an ultrasonic bath for 10 min. The extract was filtered with a spin-X centrifuge filter and then analysed on the described HPLC-MS system without further clean up.

Instrumentation

The chromatography was performed on a Symmetry C-18 column (5 μm, 4.6 × 150 mm) (Waters, Milford, USA), using a model P4000 pump and a model AS3000 autosampler (TSP, San Jose, USA). Separation was achieved using gradient elution starting with acetonitrile-water (40:60, v/v, both containing 0.05 M ammonium acetate), rising to acetonitrile-water (90:10, v/v) over 15 min. Isocratic elution with 90% acetonitrile was maintained for 10 min before the eluent was switched to 40% acetonitrile. The flow rate was 0.7 ml/min and 10 μl sample was injected. The HPLC system was coupled to the MS, an LCQ ion trap analyser, with an atmospheric pressure chemical ionisation (APCI) interface (Finningan MAT, San Jose, USA). The instrument was operated in MS full scan positive ion mode during the analysis. The ion injection time was set to 300 ms with a total of 3 micro-scans per second. For APCI a vaporisation temperature at 350 °C, a sheath gas rate at 25 units nitrogen (c 250 ml/min), an auxiliary gas rate at 5 units nitrogen (c 50 ml/min), a corona discharge voltage of 4.5 V and a capillary temperature of 200 °C were used.

Screening for metabolites

Quantitative determination of penitrem A, roquefortine C and semi-quantitative determination of penitrems B, C, D, E and F and thomitrems A and E was performed using HPLC-MS. Semi-quantitative analyses were performed using a unit response (peak area) relative to penitrem A. Solid media (rice and barley) results are reported as μg metabolite/g wet weight, while CDYE results are reported as μg metabolite/g liquid.

Results

Screening of P. crustosum isolates

Except for the Fijian and the Japanese strains, which grew slowly, the other stains grew and sporulated vigorously after incubation on rice at 20 °C for 14 days. All stains were found to produce penitrems, except for 2 Norwegian isolates, which did not produce penitrems. Among the penitrem producing isolates, thomitrems A and E, penitrem A and roquefortine C were found to be major metabolites together with 2 other unidentified metabolites exhibiting MH^+ ions at *m/z* 570 and 572 respectively (see Figure 2). One of the two non-penitrem producing isolates produced fumigaclavine A, pyroclavine and festuclavine. The content of roquefortine C in rice cultures was in the range 0.1–28 μg/g, thomitrem A 1.5–27 μg/g, thomitrem E 2.9–73 μg/g and penitrem A 0.7–58 μg/g (Table 1). Penitrems E, B and D were found in most of the isolates (Table 1) but in small amounts (0.02–2.3 μg/g) compared to penitrem A and the thomitrems A and E. Penitrems C and F were found in only a few isolates and were either not produced or produced by the other isolates at levels below the detection limit of 0.02 μg/g (Table 1).

Incubation on barley and CDYE

Results from studies of four known Norwegian penitrem producing *P. crustosum* isolates grown on

Table 1. Metabolite profiles (including roquefortine C (roq C), thomitrem E (thom E), penitrem E (pen E), unknown 570 (570), thomitrem A (thom A), unknown 572 (572), penitrem A (pen A), penitrem D (pen D), penitrem B (pen B), penitrem C (pen C) and penitrem F (pen F) in μg/g of Norwegian *P. crustosum* isolates grown on rice at 20 °C for 14 days

	roq C	thom E	pen E	570	thom A	572	pen A	pen D	pen B	pen C	pen F
801 P6	8.2	28	0.1	2.7	8.8	1.6	2.2	nd	nd	nd	nd
853 P6	28	46	0.3	0.5	21	3.9	3.9	0.06	0.1	0.05	0.1
856 P3	12	42	0.4	4.2	21	3.7	25	0.5	0.9	nd	0.1
857 P3	16	36	0.3	2.1	21	4	2.2	0.1	0.07	0.03	0.2
1098 P1	5.9	27	0.2	2.2	12	2.5	2.6	0.05	0.04	0.02	0.3
1172 F10	24	73	0.3	9.2	24	5.7	19	0.2	0.3	nd	0.1
1264 P3	14	45	0.4	6.4	23	3.9	22	0.4	0.8	nd	0.3
1323 F2	5.3	41	0.2	3.5	18	2.7	9.8	0.1	0.2	0.02	0.2
1383 F3	37	54	0.6	5.3	19	6.2	26	nd	nd	nd	nd
1564 F1	7.1	29	0.06	24	12	2.5	2.6	0.04	0.02	nd	nd
1573 F2	3.5	32	0.1	3.2	11	2.6	4.0	0.04	nd	nd	nd
1574 F8	6.5	60	0.7	6.6	26	4.8	27	0.4	0.5	nd	0.2
1578 P6	12	73	2.3	10	25	8.4	58	1.3	1.5	nd	0.3
1582 P2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
1584 F6	2.5	30	0.2	18	8.6	2.3	6.5	0.1	0.3	nd	nd
1587 P3	8.2	44	0.2	4.1	22	4.1	2.0	nd	nd	nd	0.03
1588 F6	10	51	0.2	4.5	22	4.3	2.5	0.05	nd	nd	nd
1589 P3	3.6	35	0.1	3.0	9.0	3.0	5.7	0.02	nd	nd	nd
1590 P5	5.9	61	0.5	6.8	24	5.9	26	0.6	0.7	nd	0.3
1591 P4	9.3	47	1.7	5.7	16	5.1	32	1.5	1.6	nd	0.1
1592 P5	6.2	46	0.6	6	17	5.7	24	0.03	nd	nd	nd
1601 P4	0.1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
1627 P1	6.1	57	0.3	3.8	21	4.1	14	0.1	0.4	nd	0.1
1628 P1	4.7	27	0.1	1.8	11	1.3	6	0.1	0.08	nd	0.2
1636 P1	10	34	0.2	4.1	13	3.4	11	0.1	0.2	0.03	0.07
1641 P1	5.8	39	0.2	2.4	9.2	2.3	11	0.3	0.8	0.02	0.02
1678 F6	5.7	32	0.5	1.7	6.7	1.5	12	0.4	0.7	nd	nd
1738 P2	17	47	0.6	5.1	15	7.1	16	nd	nd	nd	nd
1739 P2	12	47	0.2	3.1	12	3.3	14	0.2	0.3	nd	0.1
1778 F3	17	52	0.3	5.2	25	4.2	19	0.4	0.5	0.02	0.2
1806 F4	3.7	2.9	0.02	0.5	2.4	0.5	1.1	0.02	0.02	nd	nd
1807 F10	5.0	47	1.5	2.9	9.9	2.0	21	nd	nd	nd	0.03
1880 P2	7.5	4.2	0.03	0.4	2.1	0.4	0.7	0.02	0.04	nd	nd
2025 P1	14	67	0.3	7.1	27	4.6	20	0.2	0.3	nd	0.1
2026 F3	5.1	4.4	0.02	0.3	1.5	0.3	0.6	0.03	0.02	nd	nd
2028 P9	12	50	0.2	1.5	15	3.4	18	0.4	1.4	0.07	0.1
IBN 19848	8.8	46	0.5	9.3	27	4.5	8.5	0.2	0.1	0.03	0.2
PPRI 6859	12	22	1.3	3.6	8	4.3	11	nd	0.6	0.05	nd
CBS 313. 48	2.4	0.9	0.3	1.8	1.1	0.1	2.3	nd	nd	nd	nd
CBS 340. 59	1.8	2.9	0.8	0.4	3.4	0.08	2.2	nd	nd	nd	0.1
CBS 499. 73	11	12	1.9	1.9	5.2	0.9	4.0	0.7	0.2	nd	nd
CBS 483. 75	15	48	3.1	3.8	11	3.4	16	1.0	1.1	nd	0.2
CBS 747. 74	22	11	1.0	6.9	13	1.8	6.2	0.2	0.5	nd	nd
CBS 548. 77	9.5	22	2.5	1.5	18	2.2	13	0.09	0.05	nd	nd

nd : not detected.

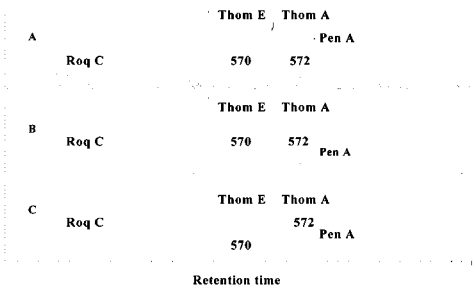

Figure 2. HPLC-MS traces of A) Norwegian (1590P5), B) Danish and C) South African isolates of *P. crustosum* grown on rice.

barley and CDYE media are reported in Table 2. Roquefortine C and penitrems A, B, C, D, E and F were found in all four isolates on both media. Thomitrems A and E were found in the extracts of all strains of *P. crustosum* when grown on barley but not when the strains were grown on the liquid CDYE media (Table 2).

Effect of temperature and incubation time on metabolite production

Metabolite profiles of one Norwegian and one South African *P. crustosum* strain incubated at different temperatures and for different times are shown in Tables 3 and 4. After one week at 15 and 25 °C the rice medium was covered by fungal growth, but at 10 °C only small colonies had been formed. The amount of metabolites increased throughout the 4 week incubation period (Tables 3 and 4). The same metabolites, roquefortine C, thomitrems A and E together with penitrem A and small amounts of penitrems B, C, D, E and F, were formed at the three temperatures. The Norwegian and South African strains of *P. crustosum* produced very similar metabolite profiles over time and temperature. Although overall production of metabolites was slightly higher in the Norwegian strain chosen for the experiment, no significant differences between the two isolates could be detected.

Effects of pH on penitrems and penitrem derivatives

Inoculation of *P. crustosum* in CYA cultures, the pHs of which were initially adjusted to values in the range 2.5 to 7, lead to the formation of penitrems A–F but no thomitrems. In each case the pH of the culture medium was close to neutral 7 days after inoculation.

Penitrem A, when added to a pH 2, 0.01 M HCl solution, showed a 10% conversion to thomitrem A. However, under these conditions (in the absence of the CYA culture matrix) thomitrem A was subsequently transformed into several other unidentified compounds.

When the pH of a week old CYA culture was adjusted to pH 3, 4 and 5, no conversion to thomitrems were detected after 72 h at pH 5. A low level (5%) of thomitrem E was detected after 24 h at pH 4 together with a corresponding decrease in the concentration of penitrem E. A 55% conversion of penitrem E to thomitrem E was observed after 24 h at pH 3, however at this pH the residual level of penitrem E was only 5% of that initial present (Figure 3). At pH 3, penitrem A also appeared to be converted to thomitrem A (15% after 24 h), however conversion occurred more slowly than was the case for penitrem E. At pH 4 the conversion of penitrem A to thomitrem A was barely detectable (1% after 24 h).

Discussion

The analytical methodology utilised in this investigation was based on that reported previously [13], modified by the use of an acetonitrile:water gradient, rather than a methanol:water gradient. This modification, described for the first time in this paper, was required to achieve adequate separation of thomitrems and penitrems.

It is well known that stored foods are liable to microbial spoilage unless special precautions are taken [14] and that the bacterial and fungal flora of food depends on many factors including type of food, temperature, storage period, water activity, pH, atmosphere and the presence of bacteria and fungi in the environment [10]. The mycoflora of stored foods, such as cereals, fruit, meat and diary products includes a variety of species of *Fusarium*, *Aspergillus* and *Penicillium*, many of which produce mycotoxins that are detrimental to human and animal health [15]. Previous investigations have shown that fungi of the genus *Penicillium* were dominant in Norwegian food wastes [16] and that *P. crustosum*, the most dominant of the identified *Penicillium* species (found in 48 of 83 Norwegian samples [16]), also produced the highest level of mycotoxins, notably penitrems.

When grown on a rice media the *P. crustosum* strains investigated here (Table 1) exhibited different metabolite profiles from those reported in studies, which utilized liquid media [2, 3, 5, 6]. None of these studies reported the presence of thomitrems A

Table 2. Concentration (μg/g) of roquefortine C (roq C), thomitrem E (thom E), penitrem E (pen E), unknown 570 (570), thomitrem A (thom A), unknown 572 (572), penitrem A (pen A), penitrem D (pen D), penitrem B (pen B), penitrem C (pen C) and penitrem F (pen F) from four selected Norwegian *P. crustosum* isolates grown on barley and CDYE at 20 °C for 14 days

	Roq C	thom E	pen E	570	thom A	572	pen A	pen D	pen B	pen C	pen F
1172 on barley	16	1.3	7.5	1.3	0.1	2.7	5.0	15	8.5	0.2	1.1
1574 on barley	41	2.9	12	2.6	0.5	2.5	15	10	9.9	0.2	1.0
1590 on barley	18	0.3	2.2	1.3	0.1	2.6	4.7	17	6.9	1.0	1.7
1592 on barley	21	0.4	4.9	3.8	0.2	1.6	1.7	30	9.4	1.3	2.1
1172 on CDYE	38	nd	19	0.9	nd	1.9	21	17	24	0.1	2.3
1574 on CDYE	51	nd	4.6	1.5	nd	2.2	20	37	22	2.6	5.2
1590 on CDYE	64	nd	12	2.3	nd	2.8	15	43	28	2.5	7.8
1592 on CDYE	56	nd	21	1.4	nd	3.7	25	50	30	1.9	4.7

nd: not detected.

Table 3. Concentration (μg/g) of roquefortine C (roq C), thomitrem E (thom E), penitrem E (pen E), unknown 570 (570), thomitrem A (thom A), unknown 572 (572), penitrem A (pen A), penitrem D (pen D), penitrem B (pen B), penitrem C (pen C) and penitrem F (pen F) from a Norwegian strain of *P. crustosum* (1590P5) incubated at 10, 15 and 25 °C with incubation times of 1, 2, 3 and 4 weeks (each value is mean of n = 3 samples) on rice

	Roq C	thom E	pen E	570	thom A	572	pen A	pen D	pen B	pen C	pen F
10 °C/1w	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
10°C/2w	2.8	29	0.3	2.9	6.9	2.3	4.3	0.2	0.4	0.07	0.09
10 °C/3w	4.3	42	0.7	5.8	20	3.9	9.8	0.2	0.4	0.5	0.2
10 °C/4w	7.0	37	0.8	6.4	17	3.5	15	0.9	0.8	0.6	0.3
15°C/1w	1.1	28	0.4	1.7	10	1.5	2.2	0.2	0.1	0.08	nd
15°C/2w	3.6	43	0.9	3.8	21	2.1	19	0.3	0.3	0.3	0.1
15°C/3w	4.4	38	1.2	5.7	30	2.1	21	0.4	0.4	1.1	0.5
15°C/4w	5.1	34	1.2	7.1	33	2.1	23	0.3	0.3	1.4	0.6
25 °C/1w	3.3	42	1.7	2.5	27	4.8	6.5	0.2	0.4	0.6	0.2
25 °C/2w	19	58	1.2	4.4	33	5.9	36	0.5	0.7	0.7	0.6
25 °C/3w	26	45	1.2	5.5	41	6.7	29	0.6	0.9	1.5	1.2
25 °C/4w	28	45	3.4	6.7	42	7.1	31	1.9	1.6	1.1	2.2

nd: not detected.

and E but most had used liquid media and not rice or barley media as in the present study. The dominant compounds detected in our rice media extracts were thomitrems A and E, penitrem A, roquefortine C and lesser amounts of the 2 unidentified substances exhibiting MH^+ ion at *m/z* 570 and 572. On the other hand, penitrem A together with penitrems B, C, D, E, F and roquefortine C [2–6] were the dominant compounds when *P. crustosum* strains were grown in liquid media such as CDYE and CYA.

One of the two non-penitrem producing Norwegian isolates, 1601P4, showed HPLC-MS peaks which exhibited ions in the MS traces that corresponded to the MH^+ ions of fumiglaclavine A (*m/z* 299) and pyroclavine or festuclavine (*m/z* 241) respectively. This strain also produced roquefortine C. Kawai et al. [17] has previously described two chemotypes of *P. crustosum*, one of which produces penitrems, while the other produces clavine alkaloids, such as fumigaclavine A, pyroclavine and festuclavine. Our observation that the metabolite profile of isolate 1601P4 corresponds to that reported by Kawai et al. [17] contrasts with that of Frisvald and Filtenborg [18] who reported that *P. crustosum* isolates always produce penitrem A and roquefortine C. The other non-penitrem producing Norwegian isolate (1582P2) afforded a series of peaks, amongst which no known metabolite could be identified. It is possible that isolate 1582P2 might have suffered from degenerative, or other changes, and lost its ability to produce penitrems. Our results in-

Table 4. Concentration (μg/g) of roquefortine C (roq C), thomitrem E (thom E), penitrem E (pen E), unknown 570 (570), thomitrem A (thom A), unknown 572 (572), penitrem A (pen A), penitrem D (pen D), penitrem B (pen B), penitrem C (pen C) and penitrem F (pen F) from a South African *P. crustosum* (PPRI6859) incubated at 10, 15 and 25 °C with incubation times of 1, 2, 3 and 4 weeks (each value is mean of n = 3 samples) on rice

	Roq C	thom E	pen E	570	thom A	572	pen A	pen D	pen B	pen C	pen F
10 °C/1w	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
10 °C/2w	1.4	12	0.08	2.3	5.9	1.9	2.6	0.02	nd	nd	nd
10 °C/3w	2.3	22	nd	6.4	22	3.3	5.8	0.2	0.3	0.2	nd
10 °C/4w	5.2	30	0.8	5.5	15	2.8	13	0.7	0.7	0.2	0.3
15 °C/1w	2.0	22	nd	2.2	17	0.9	4.3	nd	nd	nd	nd
15 °C/2w	3.4	31	0.06	3.1	12	1.7	8.1	0.08	nd	0.1	nd
15 °C/3w	5.2	37	0.3	4.8	28	1.8	12	0.4	0.3	0.9	0.4
15 °C/4w	7.1	33	1.8	6.1	28	1.7	17	0.2	0.3	1.8	0.7
25 °C/1w	4.8	40	0.9	1.2	20	3.9	5.8	nd	0.3	nd	0.03
25 °C/2w	12	34	1.3	3.6	22	4.8	12	nd	0.9	0.8	nd
25 °C/3w	18	40	1.0	6.0	34	5.7	23	0.7	0.8	1.4	1.0
25 °C/4w	19	27	1.2	5.7	33	5.8	25	1.6	1.3	0.9	2.2

nd: not detected.

dicate that the incidence of non-penitrem producing Norwegian *P. crustosum* isolates is low.

While most penicillia grow best at ambient temperatures (ca 20–25 °C), they are also known to grow and produce mycotoxins at lower temperatures [18, 19]. When cultures were grown in rice media for 1–4 weeks at 10, 15 or 25 °C, metabolites concentrations typically increased with time, until 2 or 3 weeks, after which levels tended to stabilise. Typically, no mycotoxins could be detected after 1 week at 10 °C, but after 2 weeks toxin concentrations were comparable to those obtained at 15 or 25 °C. At 10 °C typically only traces of penitrem A were detected after incubation for one week, however levels often increased tenfold after two weeks. El-Banna and Leistner [20] observed a similar trend in metabolite levels and found only small amounts of penitrem A after *P. crustosum* had been incubated for one week in various liquid media. Similarly, elevated levels of thomitrems A and E were found after one week at 15 and 20 °C, while none were detected after one week at 10 °C. Our results show that while storage of *Penicillium*-infected food at 10 °C will reduce the rate at which mycotoxins are formed, it does not inhibit their production.

Several authors have reported that the influence of physical factors such as substrate, water activity, temperatures, pH and time on the range and level of toxin produced by agar and liquid cultures [20–23]. However, few of these studies have followed the formation of a single toxin and analogues derived from it by either chemical (e.g. acid promoted rearrangements) or microbial (e.g. secondary metabolism) processes. Even if the growth of the fungi is dependent on one set of growing parameters, formation of secondary metabolites, or rearranged products, might be dependent of another. ICMSF 1996 [24] have reported that water activity is a parameter that influences the production of penitrem metabolites. Optimum production occurs when water activity is around 0.997. Below 0.990 the production of penitrem A decreases rapidly. Other authors have noted variations in mycotoxin levels when one medium is substituted for another. For example the ratio of the trichothecene mycotoxin deoxynivalenol (DON) to its 3-acetoxy analogue (3-ac-DON) when *Fusarium graminareum* is grown on grain is different from in liquid cultures [25]. In liquid culture there is usually a greater level of 3-ac-DON than is the case for DON, while on grains there is usually less than 10% of the acetylated form. This is believed to be due to diffusion of the acetylated form into the media before it can be deacetylated by the fungi.

During the course of solid media incubations the pH of the culture media typically fell from pH 7 to 4 in rice and to 5.5 in barley. This can be attributed to the production of organic acids by the rice and barley cultures [19]. The level of water available in the rice inoculates was 5 times higher than that available in the barley inoculates and may therefore account for the lower pH (greater acidity) of the aged rice inoculates. Since it is well known that penitrems are unstable at low pH [26] we investigated the possibility that the increased acidicity of rice culture medium

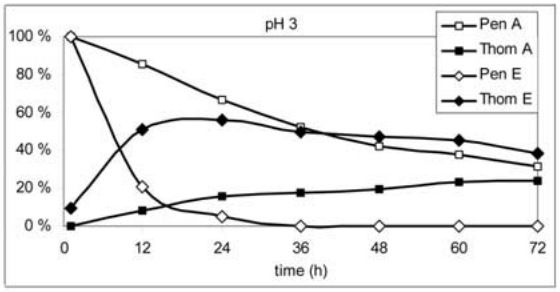

Figure 3. Conversion (%) of penitrems A and E to thomitrems A and E determined following adjustment of CYA medium to pH 3.

might promote the conversion of penitrems A and E to thomitrems A and E. Experiments in which, a week old (c pH 7) liquid culture, which contained only penitrems was diluted with acetonitrile and acidified to pH 3, 4 or 5, and monitored over 12 h periods showed that penitrems A and E were partially converted to thomitrems A and E (Figure 3). Penitrem A was converted to thomitrem A more slowly than was the case for the conversion of penitrem E to penitrem E. Greater conversion was observed at pH 3 than at pH 4. During the 72 h trial, no conversion of penitrems to thomitrems was observed at pH 5 or 7. Penitrems B, C, D and F showed little conversion to peaks, which possibly corresponded to the corresponding thomitrems. Those observations can be interpreted as indicating that the presence of a chlorine atom in penitrem A (but not penitrem E) reduces the rate at which penitrem A is converted to thomitrem A. The ratios of thomitrem A to penitrem A, and thomitrem E to penitrem E determined at pH 3 over a 72 h period (see Figure 3) were comparable to those observed for the rice cultures (Table 1) at pH 4 over a 21 day period.

A standard solution of penitrem A in acetonitrile:water acidified to pH 2 with 0.01 M HCl acid solution afforded only a moderate level of thomitrem A (c 5% after 12 h) while the amount of penitrem A decreased by 90%. Under these conditions thomitrem A appeared to be transformed into several other unidentified compounds which exhibited HPLC-MS characteristics (mass spectra and retention times) which differed for those observed for minor peaks in rice cultures extracts.

Inoculation of *P. crustosum* on CYA with pH's in the range 2.5 to 7 lead to the formation of penitrems A–F but not thomitrems. The pH of the CYA solutions increased during the inoculation period and in all cases was close to neutral after 7 days, implying that fungi produce substances which reduce the acidity of the pH adjusted CYA media (initially pH 2.5, 4 or 5.5). The absence of thomitrems A and E in these experiments is consistent the stability trial results which showed that penitrems A and E were not converted thomitrems A and E in these experiments were the final pH was close to 7.

Conclusions

It is apparent that in circumstances where the pH of the media may fall (become more acidic) during growth, or storage, conversion of penitrems A and E to the corresponding thomitrems is likely. Whether thomitrems should be classified as natural products (metabolites produced by cultures of *P. crustosum* fungi grown under defined conditions) or as penitrem artefacts (substances derived under specified conditions from precursor metabolites) is of secondary importance given the occurrence of thomitrems (Table 1) in low acidity food and grain matrices which may be encountered in natural environments. Given our demonstration that thomitrems A and E can be consistently and reliably produced from a range of Norwegian and international isolates, or pH adjustment of aged liquid CYA cultures, and their likely occurrence in wild (natural) fungal cultures growing in acidic environments, it is important that in the near future sufficient quantities of thomitrems A and E are isolated and their toxicities established, since it is presently not known if they are more or less toxic than the penitrems from which they are derived.

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