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Determination of *Penicillium roqueforti* toxin by reversed-phase high-performance liquid chromatography☆

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ABSTRACT

A method for the detection and quantification of *Penicillium roqueforti* toxin (PRT) using reversed-phase high-performance liquid chromatography has been established. The limit of quantitation of this method was 3 ng of PRT, while the limit of detection was 2 ng of toxin. The precision of the analysis based on numerous runs was good. Retention times for PRT were highly reproducible with an average coefficient of variation of about 1.6%. Analysis of PRT in liquid and solid samples showed no interference of the sample matrix. The accuracy of the method was 98.6%, with mean PRT recoveries of 96.8%, and 100.4% for the spiked culture medium and blue cheese extracts, respectively.

INTRODUCTION

Penicillium roqueforti toxin (PRT) is a toxic fungal metabolic first isolated from mouldy grains and corn silage [1] and then subsequently from cultures of *P. roqueforti* including some of those which are used in blue-veined cheese production [2,3]. PRT is lethal for rats and mice with LD_{50} values of about 6–15 mg/kg by intraperitoneal administration [4]. In addition, PRT was found to be carcinogenic for rats [5,6] as well as mutagenic for *Salmonella typhimurium* [7].

A number of thin-layer chromatographic methods are available for the detection of PRT in a variety of media [8]. These methods are mainly used for identification and semi-quantitative determination of the PRT. More recently a normal-phase high-performance liquid chromatographic (HPLC) technique has been developed to measure the concentration of this toxin and other *P. roqueforti* metabolites in culture broths [9]. It has been used to follow the transformation of eremofortin C into PRT with a detection limit of 10 ng for the latter toxin [10–12]. In addition, reversed-phase HPLC analysis of PRT has also been briefly reported [13]. This technique, however, allows for detection of the toxin at a level of more than 50 ng. Although most of the above methods were successfully used for the detection of PRT in culture broths, determination of this toxin in cheese imposed problems due to its reactivity with proteins and amino acids as well as being influenced by the sample matrix [2,14].

In this paper we describe a method for the detection and determination of PRT using reversed-phase HPLC, which is more sensitive than chromatographic methods reported earlier. The feasibility to employ this method for the determination of PRT in culture broths and blue cheese was also investigated.

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EXPERIMENTAL

Materials

Reference PRT was obtained from Sigma (St. Louis, MO, USA). High-performance thin-layer chromatography (HPTLC) silica gel 60 F-254 aluminium plates were purchased from BDH (Toronto, Canada). A PRT-producing strain of *P. roqueforti* (ATCC No. 10110) was obtained from the American Type Culture Collection (Rockville, MD, USA) while Danish blue cheese was purchased from a local supplier. All solvents were of HPLC grade.

Chromatography

The HPLC method was developed on a Waters ALC 204 liquid chromatograph. The system consisted of a Model 6000A solvent delivery system, a Model 440 absorbance detector equipped with a 254-nm filter and a U6K universal liquid chromatography injector.

Chromatographic analyses were carried out on a pre-packed LiChrosorb reversed-phase C_{18} column (240 mm × 4.0 mm I.D.) of particle size 10 μm (E. Merck, Darmstadt, Germany). The signal from the detector was recorded by an SP4290 integrator (Spectra Physics) set at an attenuation of 8. A volume of 20 μl was injected for all samples. Mobile phases consisting of methanol–water in the ratios 70:30 (v/v) and 65:35 (v/v) were used for HPLC analysis. All runs were carried out at a flow-rate of 1.0 ml/min.

The presence of PRT in samples prior to reversed-phase HPLC analyses was assessed by analytical thin-layer chromatography (TLC) using chromatoplates of silica gel F-254 with methanol–chloroform (5:95, v/v) as the eluent [1].

Standard preparation

The reference PRT (Sigma) was further purified by normal-phase TLC. Toxin was dissolved in chloroform (0.5 μg/μl) and spotted on the TLC plate. The plate was developed using a mixture of methanol and chloroform in the ratio of 5:95 (v/v). The PRT spot, having an R_F value of 0.80, was located under short-wavelength UV light (254 nm) and clearly marked. The silica containing the PRT was carefully scraped from the plate, transferred to a vial and resuspended in 2.0 ml of methanol in order to extract the toxin. The resulting suspension was filtered through Whatman No. 4 paper. The PRT was extracted from silica two more times with a total of 2.0 ml of methanol. The three filtrates were combined and evaporated to dryness at room temperature under a stream of nitrogen, yielding a crystalline PRT standard used in further studies. Purity of the PRT standard was first determined using a diode-array spectrophotometer (Hewlett-Packard). The PRT crystals dissolved in 1.0 ml of methanol exhibited a single peak at 248 nm corresponding to its absorption maximum described by other workers [3]. In addition, the ^1H NMR spectrum (300 MHz) of the purified toxin was measured in $^2HCl_3$ using a NMR spectrometer (Model AM 300, Bruker Spectrospin, Canada). The spectrum was identical to the PRT spectral data obtained by Wei *et al.* [14]. Finally, the effectiveness of the purification procedure was assessed by reversed-phase HPLC (Fig. 1). The chromatography yielded a single peak indicating a high purity of the PRT standard. The concentration of the toxin dissolved in methanol was measured spectrophotometrically at 248 nm (ε = 9000) [3].

Standard curves were prepared by making a series of dilutions of PRT in methanol ranging in concentration from 0.15 to 30 ng/μl. Samples were ana-

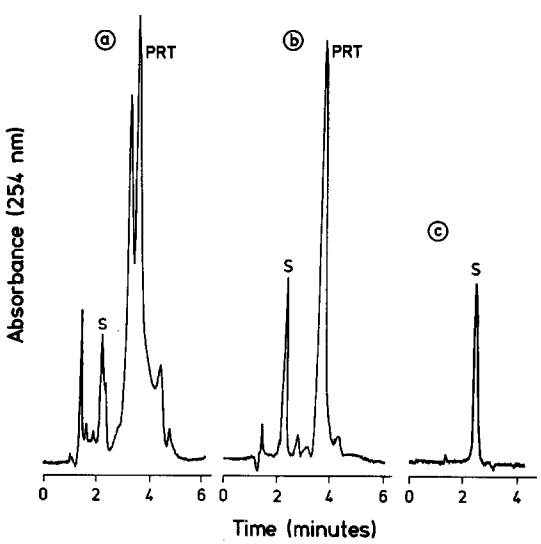

Fig. 1. Chromatograms of PRT standard: (a) 200 ng of an impure mixture containing PRT, (b) 60 ng of PRT after TLC purification and (c) 20 μl of methanol. Eluent, methanol–water (65:35, v/v); flow-rate, 1.0 ml/min. Peak S = methanol.

lyzed chromatographically as described above and the peak height and peak area were plotted against the corresponding concentration.

Sample preparation

To assess the suitability of reversed-phase HPLC for the determination of PRT in different samples, analysis of this toxin in culture broths (preparation 1) and cheese (preparation 2) was conducted.

Preparation 1. PRT was produced using the strain of *P. roqueforti* ATCC No. 10110, according to the procedure of Wei *et al.* [1]. The mould was grown for 14 days at room temperature in a culture medium containing yeast extract and sucrose (YES, Difco). Then, the mycelium was removed from the culture broth by paper filtration and the broth (100 ml) was extracted with chloroform to yield a crude sample containing PRT. The presence of PRT was confirmed by TLC as described earlier in this paper. Crude samples from two separate fermentation batches were evaporated to dryness, each dissolved in 1.0 ml of methanol, and used for HPLC analysis.

Preparation 2. Purified PRT standard (0.5 mg), dissolved in 1.0 ml of methanol, was added to 40 g of Danish blue cheese to give a final concentration of 12.5 mg/kg. The cheese was immediately homogenized in a Waring blender with 250 ml of methanol–water (55:45, v/v) and 150 ml of hexane for 5 min at high speed and then divided into two equal portions. Each portion was centrifuged at 1000 *g* at 4°C for 10 min. After removing the hexane layer, the methanol–water part was vacuum-filtered through Whatman No. 3 paper and the resultant filtrate was extracted with two 60-ml volumes of chloroform. The two chloroform extracts from each portion were pooled and evaporated to dryness using a rotary evaporator. The dried extract was dissolved in 1.0 ml of methanol, 10 μl were withdrawn, diluted 40-fold with methanol, and immediately analyzed by HPLC as described earlier. The remaining extract was stored at −5°C for 24 days and the PRT content was periodically analyzed by HPLC. Cheese used for this experiment was free of PRT as assessed by TLC. Part of cheese was extracted exactly as described above, and the extract was used as a control sample (see Fig. 4b).

Accuracy assessment

The accuracy of the HPLC method for PRT quantification was determined by an external standard method. Culture medium and blue cheese samples free of PRT were extracted with chloroform, evaporated to dryness, dissolved in methanol and spiked with PRT over the concentration range 1.0–8.3 ng/μl (20–166 ng per injection). Recoveries of PRT from extracts were assessed by HPLC using peak height measurements. A two-sample *t*-test was used to estimate the significance of the differences between known and measured values of PRT.

RESULTS AND DISCUSSION

The PRT retention times for two solvent systems containing methanol and water are shown in Table I. The average retention time for PRT using methanol–water in a 70:30 (v/v) ratio was 3.31 min based

TABLE I

REPRODUCIBILITY OF RETENTION TIME OF PRT AS ANALYSED BY REVERSED-PHASE HPLC

Trial No.	Solvent[a] ratio	n^b	Retention time (min)			C.V. (%)
			Range	Mean	S.D.	
1	70:30	24	3.15–3.44	3.26	0.11	3.37
2	70:30	23	3.31–3.39	3.34	0.02	0.60
3	70:30	32	3.25–3.41	3.32	0.05	1.51
4	65:35	67	3.60–3.84	3.70	0.05	1.35
5	65:35	17	3.55–3.84	3.69	0.12	3.25
6	65:35	27	3.92–3.96	3.93	0.01	0.25
7	65:35	41	3.75–3.89	3.84	0.03	0.78

[a] Methanol–water (v/v).
[b] Number of injections.

TABLE II

REPRODUCIBILITY OF HPLC PEAK AREA AND PEAK HEIGHT FOR PRT USING 65:35 (v/v) METHANOL–WATER AS SOLVENT SYSTEM

PRT injected (ng)	n^a	Peak area or height			C.V. (%)
		Range	Mean	S.D.	
Area					
55.0	20	125 952–218 709	163 445	21 628	13.2
28.0	12	60 017–92 814	72 088	9860	13.7
Height					
55.0	23	5525–6512	5899	388	6.58
28.0	6	3661–4023	3831	123	3.22

[a] Number of injections.

on three separate trials over 4 days. Coefficients of variation (C.V.) ranged from 0.06 to 3.37%. Methanol–water in a 65:35 (v/v) ratio, as the eluting solvent resulted in an average retention time of 3.79 min for PRT, based on four trials over a 4-day period. C.V.s with this solvent ranged from 0.25 to 3.25%. The PRT retention times for both solvent systems were highly reproducible. However, the methanol–water in ratio 65:35 (v/v) allowed for longer retention of the toxin on the column and, hence, better resolution of the PRT peak from the initial solvent peak.

The precision and reproducibility of the peak height and the peak area were analyzed by injecting a volume (20 μl) containing either 55 or 28 ng of the purified standard, over a 2-day period. The C.V.s for the peak area measurements were 13.2 and 13.7%, respectively. Reproducibility of peak heights for the same injected amounts were better with C.V.s of 6.58 and 3.22%, respectively (Table II). Consequently, further determination of PRT by HPLC was based on the peak height measurements.

Total PRT recoveries and results of a Student t-test are shown in Table III. The accuracy of the method for PRT determination was 98.6%, with mean recoveries of 96.8%, and 100.4% for spiked culture medium and blue cheese extracts, respectively. No significant difference (at a 95% confidence level) was found between the means of the standard PRT and the recovered PRT from spiked culture medium or cheese samples over the concentration range tested. The only exception was the

TABLE III

ACCURACY OF REVERSED-PHASE HPLC TO DETECT PRT IN CULTURE MEDIUM AND BLUE CHEESE SAMPLES

Amount of standard PRT[a] (ng)	Culture medium samples			Blue cheese samples		
	Mean PRT recovered[a] (ng)	Student t-value[b]	Total recovery (%)	Mean PRT recovered[a] (ng)	Student t-value[b]	Total recovery (%)
166.0	158.4 ± 3.96	2.24	95.4	151.6 ± 5.60	3.84	91.3
125.0	111.7 ± 7.83	3.14	89.4	126.6 ± 18.5	0.15	101.3
84.0	81.1 ± 6.35	0.78	96.5	84.0 ± 9.70	0.00	100.0
62.0	64.7 ± 1.83	2.02	104.4	60.1 ± 15.0	0.22	96.9
50.0	45.5 ± 0.66	3.24	91.0	50.0 ± 7.00	0.00	100.0
20.0	20.8 ± 0.65	1.50	104.0	22.6 ± 0.79	4.39	113.0

[a] Based on triplicate injections.

[b] Two-sample t-test made at a 95% confidence level where $t_{\alpha=0.05} = 4.30$.

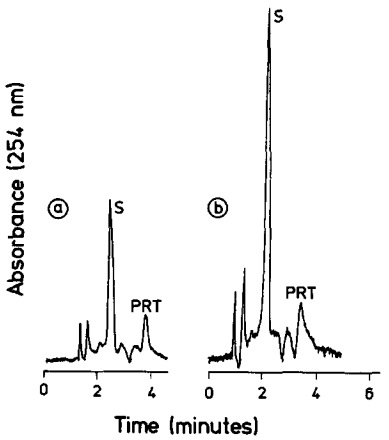

Fig. 2. Chromatograms of PRT standard: (a) 3.0 ng at attenuation 8 and (b) 2.0 ng at attenuation 4. Eluent, methanol–water (65:35, v/v); flow-rate, 1.0 ml/min. Peak S = methanol.

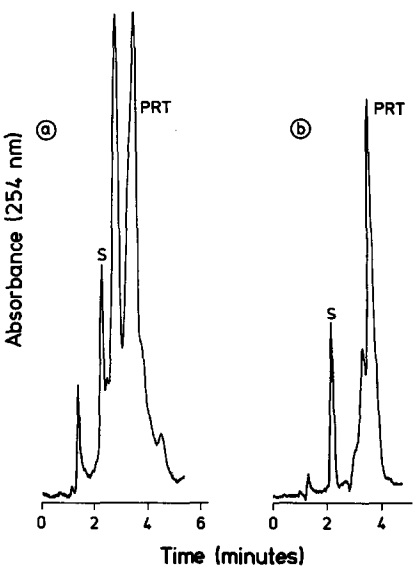

Fig. 3. Chromatograms of crude PRT extracts obtained from two separate fermentation batches of *P. roqueforti* in 100 ml of YES culture medium. Eluent, methanol–water (65:35, v/v); flow-rate, 1.0 ml/min. Peak S = methanol.

spiked cheese extract at the lowest concentration level of 20 ng PRT where the *t*-value was slightly higher (4.39) than the tabulated value (4.30).

The limit of detection of the method was determined with the integrator set at an attenuation of 8. The highest sensitivity setting of 1 resulted in a very noisy baseline that interfered with the interpretation of the chromatogram and, hence, a more appropriate setting of 8 was chosen. With the parameter at this setting, PRT could be measured at a concentration as low as 3 ng per run (Fig. 2a). Furthermore, at an attenuation setting of 4, an amount of 2 ng of toxin could be detected (Fig. 2b). This sensitivity was better than those ereported earlier by other HPLC methods [9,13].

In order to carry out the quantitative analysis, it was necessary to study the linearity of the detector response (peak area and peak height) with respect to concentrations of PRT. The linearity was evaluated by injecting 20 μl of increasing concentrations of the toxin standard, so that the injected amounts of PRT ranged from 3 to 600 ng. Regression analysis of the resulting calibration curves, obtained from three trials each ranging from 3 to 70 ng of PRT, indicated that the relationship between both peak height or peak area and the amount of PRT injected was linear over the tested concentration range, with correlation coefficients of 0.93 and 0.92, respectively. For the broader range of PRT concentration (3–600 ng) the correlation coefficient for peak area and the amount of PRT injected was 0.97.

The selectivity of the developed reversed-phase HPLC method was assessed by the determination of PRT in samples each having a different matrix: *P. roqueforti* culture medium and blue-veined cheese. Fig. 3 shows chromatograms of crude PRT extracts obtained from two different fermentation batches. Both of these indicate the presence of the toxin produced by *P. roqueforti* during the fermentation process. Presence of the toxin was verified by spiking the extracts with standard PRT. Distinct PRT peaks in both chromatograms indicate that other extractable components do not interfere with the detection of the toxin. With the aid of the calibration curve (peak height *versus* concentration), a total PRT concentration of 0.6 and 2.3 mg/ml was detected in the first (Fig. 3a) and in the second (Fig. 3b) extract, respectively.

A typical chromatogram of PRT recovered from cheese is shown in Fig. 4a, while Fig. 4b shows a chromatogram of the extract obtained from cheese prior to the addition of the toxin. Also in this case, the PRT peak was distinct (Fig. 4a) and detection of

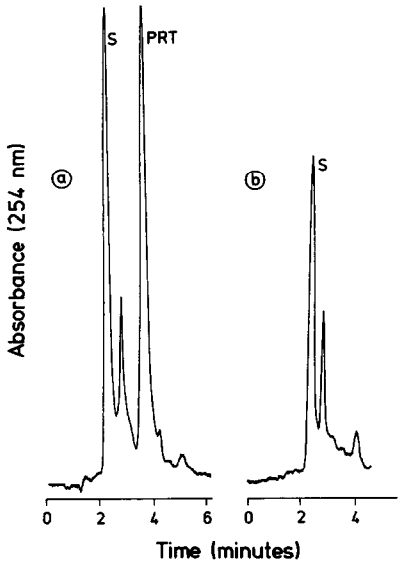

Fig. 4. Chromatograms of (a) crude PRT recovered from PRT-spiked blue-veined cheese (0.5 mg per 40 g) and (b) a control cheese extract. Eluent, methanol–water (65:35, v/v); flow-rate, 1.0 ml/min. Peak S = methanol.

the toxin was not influenced by other components present in the extract obtained from the cheese matrix (Fig. 4b). Numerous analyses of PRT in methanol extract of cheese stored at −5°C for 24 days resulted in an average retention time of 3.97 min, with C.V. of 2.5%. This indicates good reproducibility of this parameter. Although the retention time was slightly higher than that of the standard PRT (3.79 min), presence of the toxin was verified by spiking the sample with the standard.

The determination of PRT allowed for the assessment of the recovery of toxin added to cheese and subsequent stability analysis of the recovered PRT in methanol extract stored for 24 days (Table IV). Although the extraction procedure for cheese was designed to be simple and as rapid as possible, a mean recovery of only 43.6% PRT was obtained as determined with the aid of the peak height calibration curve. This is not unexpected since the instability of PRT in blue cheese and its low yield of recovery has been reported by other [15]. The toxin forms *P. roqueforti* imine with ammonia and reacts with neutral and basic amino acids present in cheese. It has been reported that the aldehyde group of the toxin is the moiety that reacts with the amino group

TABLE IV

STABILITY OF PRT IN METHANOL EXTRACT OF BLUE CHEESE AS DETERMINED BY REVERSED-PHASE HPLC

Based on triplicate runs and analysis performed using HPLC peak height.

Time after extraction (days)	Amount of PRT in extract[a]	
	μg/μl	%
0	0.218	43.6
1	0.217	43.4
4	0.219	43.8
5	0.194	38.8
11	0.135	27.0
17	0.125	25.0
20	0.122	24.4
24	0.119	23.8

[a] PRT was added to cheese at 12.5 mg/kg and extracted as described under Experimental.

of the amino acids forming Schiff bases [15]. Moreover, PRT may react with the ε-NH_2 group of lysine [16] and sulfhydryl group of cysteine [17].

Studies on the stability of PRT in methanol extract shows that the toxin was relatively stable over approximately 4 days. However, prolonged storage at −5°C for up to 24 days resulted in a 55% decrease in the amount of PRT present in the cheese extract (Table IV). Subsequent loss of PRT may be due to its reactivity with compounds such as tryptamine or other primary amines extracted from blue cheese that are present in concentrations of up to 2.3 mg/g [15,18]. This is supported by the fact that the standard PRT in pure methanol (0.01 μg/ml) was stable at −5°C for at least 1 month.

The developed reversed-phase HPLC method can be used for the determination of PRT produced by *P. roqueforti* in culture broth extracts. Moreover, because of its specificity and sensitivity, the HPLC method is suitable to detect the toxin in blue cheeses.

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