Identification of the Main Degradation Products of Patulin Generated Through Heat Detoxication Treatments

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ABSTRACT

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The chemical stability of patulin (PAT) was studied in model aqueous media at different temperatures and pH values in the presence and absence of sulfites. At pH 6, 50% was degraded within one hour at 100°C. At lower pH the detoxication efficiency was strongly reduced. The compound 3-keto-5-hydroxypentanal was shown to be the main degradation product of patulin. As the hemiacetal function has to be transformed into an aldehyde before retroaldolization and lactone hydrolysis, sulfites, as expected, improved detoxication, especially at high pH. At pH 7 in the presence of 50 ppm sulfite, PAT was completely degraded within 3 h at 25°C.

Keys words: detoxication, mycotoxin, patulin, pH, sulfites.

INTRODUCTION

Patulin (4-hydroxy-4H-furo-(3,2c)-pyran-2-(6H)-one, PAT) (Fig. 1) is a fungal toxin of *Penicillium expansum* which mainly contaminates apple products and cereals (IARC group 3). The World Health Organisation (WHO) recommends a maximum permitted level of 50 µg/L for apple juice and also the European Commission recently adopted a maximum permitted level of 50 µg/kg for patulin in a range of foodstuffs mainly derived from or containing apples.

According to Lovett and Peeler[13], PAT is quite stable in aqueous solution at 105-125°C in the 3.5–5.5 pH range (McIlvaine's buffer). PAT degradation increases as the PAT concentration decreases and the pH increases[6]. Brackett and Marth[2], using Sorensen's phosphate buffer at 25°C, found the half-life of PAT to be 64 h at pH 8 versus 1310 h at pH 6 (apparent 1st-order reaction). Therefore, heat treatment (pasteurization) and storage cannot completely inactivate PAT in apple juice or ciders[9,10]. Kadakal and Nas[10] report, however, that thermal treatment of apple juice at 100°C for 20 min followed by evaporation leads to 30% degradation. Surprisingly, 4 months of dark storage also appears to decrease (by 50%) the PAT level in apple juice[1].

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PAT shows faster degradation in the presence of SH-containing compounds (cysteine, gluthatione)[6,7,12]. Scott and Somers[18] report that in a model thiol gluthatione medium, PAT decomposes even at pH values as low as 2.3 or 3. According to Ciegler[4], the high cysteine content of some foods (e.g., cheese) may interact with PAT to render it inactive. Because of its electrophilic character, PAT can react (cross-link) with cellular nucleophiles such as amino acids (cysteine and also lysine and histidine) and proteins (BSA, tubulin)[7].

Aytac and Acar[1] have shown PAT to be unstable in the presence of 100 ppm SO_2 (decrease from 6000 to 200 ppm PAT after 4 months at room temperature). In this way, PAT could be efficiently degraded in wet corn, Durham wheat, or sorghum)[16]. Addition of 100 ppm SO_2 to grape wine halved the PAT concentration within 15 min[15]. Yet according to Burroughs[3], more than 200 ppm sulfite is required to reduce PAT efficiently in apple juice. Levels as high as 2000 ppm are necessary to degrade 90% of the PAT in 2 days (initial level = 150 ppm).

Addition of 500 ppm ascorbic acid, sodium ascorbate, or both at pH 7.5 has emerged as another advantageous way to degrade PAT in juice from apples contaminated with *Penicillium expansum* (decrease from 6000 to 60 ppm PAT after 4 months at room temperature)[1,2]. Drusch et al.[5] recently showed that the degradation of patulin is induced by either hydroxyl radicals or other free radicals generated by the oxidation of ascorbic acid to dehydroascorbic acid. According to Lai et al.[11], PAT can be totally destroyed within three weeks. Brackett and Marth[2] report that the rate of decomposition increases with the ascorbate concentration.

McKenzie et al.[14] have shown PAT solutions (32 µM) treated with ozone gas to be completely detoxified within 15 sec. Gamma irradiation is another way to eliminate PAT in apple juice (2.5-kGy dose for 2 ppm PAT[19]). PAT degradation is proportional to the γ dose[19].

The aim of the present work was to identify the degradation products issued from the chemical detoxication of PAT. The influence of pH and sulfites will be also discussed.

EXPERIMENTAL

Chemicals

Acetonitrile and methanol were purchased from Romil (UK). Patulin, butan-2-one, anhydrous ether, sodium ethoxide, 4-hydroxy-2-butanone, and ethyl formate were

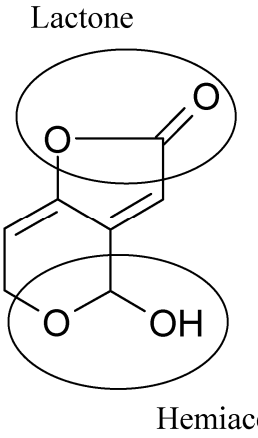


Fig. 1. Structure of patulin (PAT).

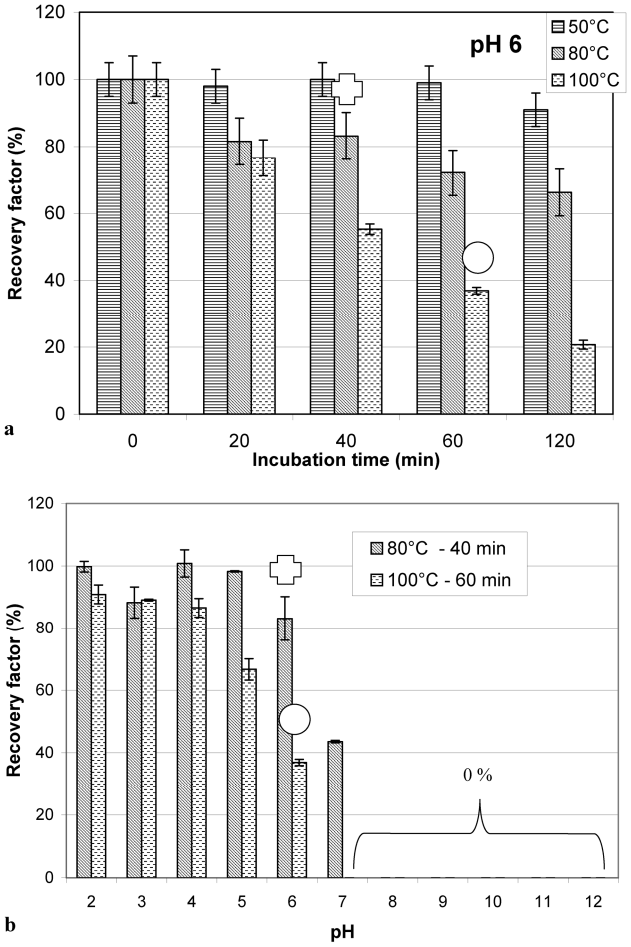


Fig. 2. Influence of (a) incubation time (50°C, 80°C, and 100°C) and (b) pH (80°C for 40 min or 100°C for 60 min) (initial PAT concentration = 4 ppm).

from Sigma-Aldrich (Belgium), and sodium hydroxide (NaOH) from Baker (Holland). Absolute ethanol, hydrochloric acid (HCl), di-sodium hydrogen phosphate dihydrate, and mono-hydrated citric acid were purchased from Merck-VWR (Belgium), sodium bisulfite from Janssen Chimica (Belgium) and tri-sodium dodecahydrate from UCB (Belgium). Liquid nitrogen was supplied by Air Liquide (Belgium). Aqueous solutions were made

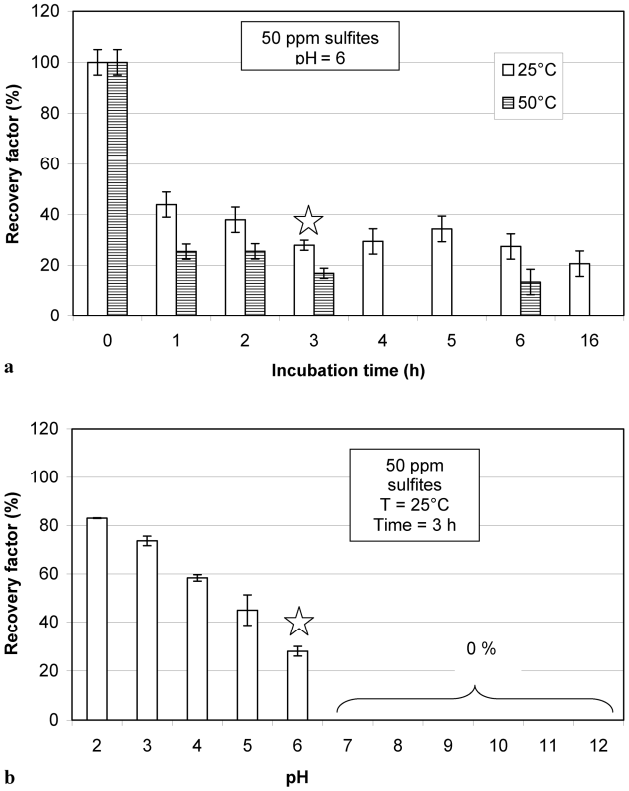


Fig. 3. Influence of the spiking of 50 ppm sulfites (a) at 25°C and 50°C and (b) at different pH (25°C, 3 h) (initial PAT concentration = 4 ppm).

with Milli-Q (Millipore, USA) ultrapure water (resistance = 18 MΩ).

Preparation of pat solution

PAT solution was prepared by dissolving 10 mg of crystalline PAT in 50 mL ethanol[17]. The accurate concentration (close to 200 ppm) was determined by spectrophotometric absorption at 275 nm (ε_{PAT} = 14609 $L.mole^{-1}.cm^{-1}$). This solution was stored in an amber vial at –20°C to avoid any degradation.

Influence of incubation time and temperature

A 50 µL aliquot of PAT solution was introduced into different amber vials and evaporated at 35°C under a nitrogen atmosphere. These PAT residues were then dissolved in 2.5 mL pH 6 McIlvaine's buffer (final concentration close to 4 ppm). After dissolution (vortexing), vials were incubated in a water bath at 50°C, 80°C, or 100°C for 20, 40, 60, or 120 min. One vial, kept as a reference (no treatment), was immediately injected.

Influence of pH

PAT residue was dissolved in 2.5 mL pH 2–12 McIlvaine's buffer and incubated for 40 min at 80°C or for 60 min at 100°C (water bath). For each pH, one vial, kept as a reference (no treatment), was immediately injected.

Influence of sulfites

PAT residue was dissolved in 2.5 mL pH 6 McIlvaine's buffer. A 100 µL sulfite solution (203 mg $NaHSO_3$/100

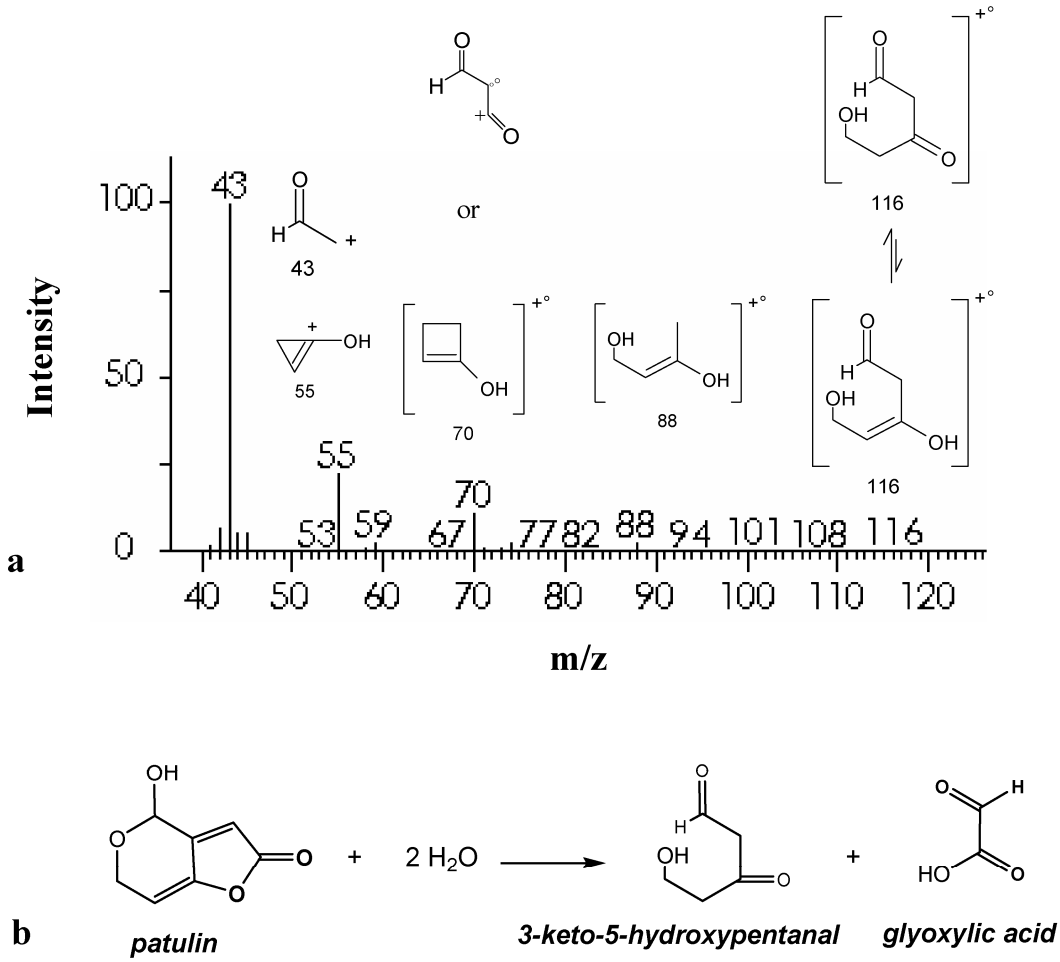


Fig. 4. (a) GC-MS spectrum of the PAT degradation product (degradation after 80°C, pH 8 for 40 min) and (b) hypothetical mechanism for PAT degradation.

mL water, final SO_2 concentration = 50 ppm in each vial) was added before heat treatment. Mycotoxin degradation was studied for 16 h at 25°C (room temperature) or 50°C (water bath). Assays were also carried out for 3 h at 25°C, at different pH values (2–12; McIlvaine's buffers).

High performance liquid chromatography analysis of PAT (HPLC-UV)

A Waters 600 Pump and Controller system (Waters, Belgium), a Shimadzu CR4A integrator (Shimadzu, Belgium), and a Waters 2487 UV detector (Waters, Belgium) were used for PAT quantification (at 275 nm). PAT was separated on a 5-µm Nucleosil RP C18 column, 250 × 4.6 mm i.d. (Alltech, Holland) at 25 °C. Separations were carried out at a flow rate of 1 mL/min with a linear gradient from A (water) to B (acetonitrile). Gradient elution was 5% B, 0–5 min; 5–95% B, 5–25 min; 95% B, 25–40 min isocratic. Aqueous PAT samples were injected with the 5-µL Waters 600 7725i Rheodyne loop (Waters, Belgium).

Patulin degradation product analysis

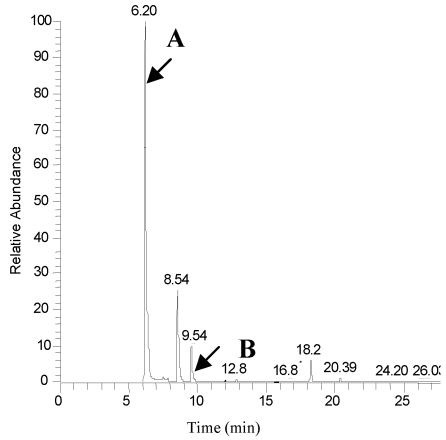
PAT solution (0.5 mL) placed in different vials was evaporated under a nitrogen atmosphere and dissolved in 2 mL double-distilled water. Aqueous PAT solutions were degraded at 80°C and pH 8 (adjusted with NaOH) for 40 min. The degraded aqueous samples were adjusted to pH 5 with NaOH and then extracted with diethylether (3 × 2 mL). The organic fraction was dried with sodium sulfate, concentrated to 0.25 mL at 39°C in a Dufton vial, and injected into the GC-FID and GC-MS setups. In order to confirm the high polarity of the PAT degradation product, the same experiment was further conducted with butan-2-one instead of diethylether. In this case, the intensity of the GC peak at RI = 874 strongly increased.

Gas chromatography-mass spectrometry analysis (GC-MS)

The gas chromatograph was a Trace GC (Finnigan Mat, USA) equipped with a split-splitless injector, and an MS detector linked to a computer with the Xcalibur software version 1.2 (Finnigan Mat). PAT degradation products were separated using a 50 m × 0.32 mm i.d., wall coated open tubular (WCOT) non-polar CP-Sil5-CB capillary column with a 1.2-µm film thickness (Varian, Belgium). The oven temperature was programmed to rise from 36°C to 85°C at 20°C/min, then to 145°C at 1°C/min, then to 250°C at 3°C/min and finally to remain constant at 250°C for 30 min. The injected sample volume was 1 µL. The injection temperature, split flow, and split-

(a) $NaOC_2H_5 + \underset{A}{HO\text{-}CH_2\text{-}CH_2\text{-}CO\text{-}CH_3} + HCO_2CH_2CH_3 \rightarrow \underset{B}{HO\text{-}CH_2\text{-}CH_2\text{-}CO\text{-}CH_2\text{-}CHO}$

(b)



(c)

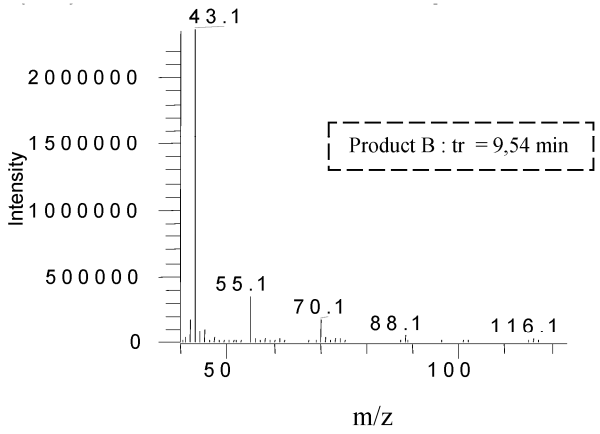


Fig. 5. Synthesis of 3-keto-5-hydroxypentanal: (a) chemical pathway, (b) GC chromatogram and (c) mass spectrum.

less time were respectively 250°C, 20 mL/min, and 1 min. The retention index (RI) was calculated by using n-alkanes as references. MS analyses were carried out with a Trace MS quadrupole mass spectrometer (Finnigan Mat, USA). Electron impact mass spectra were recorded at 70 eV (2.45 scans per second) with a 40–400 amu range.

3-Keto-5-hydroxypentanal chemical synthesis

The β-ketoaldehydes are known to be able to trimerize in acidic medium or to polymerize into a tarry product in alkaline solution. We thus adapted the synthesis of Franck and Varland[8] by using a very dilute solution and diethyl ether as a solvent in which sodium ethoxide is insoluble.

In a 100-mL dried Schott flask were placed 40 mL dry ether, 2 drops of 4-hydroxybutan-2-one, 2 drops of ethyl formate, and 12 mg sodium ethoxide. A magnetic bar was added and the flask was stoppered. After 1 h of mixing, the insoluble sodium ethoxide was allowed to settle for 10 min and the supernatant ether solution was subjected to GC-MS analysis. Control experiments in which any single constituent was omitted showed no peak at 9.54 min.

RESULTS AND DISCUSSION

Influence of incubation time and temperature on PAT detoxication

PAT (Fig. 1) stability was first tested at 50°C, 80°C, and 100°C for 20, 40, 60, and 120 min (pH = 6). The PAT concentration did not significantly decrease at 50°C but at 100°C, decomposition was half-complete after 40-60 min (Fig. 2a). Recovery was close to 80% after treatment at 80°C for the same time. Two heat treatments (one indicated by a cross, the other by a circle) were selected as starting points for investigating the influence of pH.

Influence of pH on PAT detoxication

Degradation tests were first carried out at 80°C for 40 min, with the pH ranging from 2 to 12. As depicted in Fig. 2b, PAT was stable in acidic media and began to decompose at pH 6 (83% recovery; 44% at pH 7). Farkas and Schreiner[6] have also mentioned that the degradation level increases with increasing pH. PAT decomposition ap-

peared not to be reversible (no higher recovery after subjecting the pH 7 medium containing the degradation product to a 40 min incubation at pH 4 and 80°C). The same experiments were further conducted under more drastic conditions (100°C, 60 min) (Fig. 2b). In this case, PAT was markedly degraded in acidic media (66% and 0% recovery at pH 5 and 7, respectively).

Influence of sulfites on PAT detoxication

The impact of 50 ppm SO_2 equivalent was investigated at room temperature (25°C) and at 50°C for different incubation times (0–16 h) (pH = 6). Fig. 3a shows that 1 h at room temperature was sufficient at cause the PAT level to decrease (45% recovery, 28% after 3 h). At 50°C, 26% recovery was recorded after 1 h, versus 100% in the absence of sulfite.

The effect of pH was also investigated (Fig. 3b). Again, the higher the pH, the better the degradation. With 50 ppm SO_2 equivalent, 3 h was sufficient to reach total destruction at room temperature, pH 7.

GC-MS analysis of PAT degradation products

Diethylether extraction applied to the 'degraded patulin' medium (80°C for 40 min, pH = 8) enabled us to detect a new GC peak at 9.54 min (retention index = 874 on CP-Sil5 CB). The corresponding mass spectrum (Fig. 4a) was very similar to that of propanal-3-hydroxyacetate, according to the MS library. An isomer, 3-keto-5-hydroxypentanal, was suspected. This aldehyde might result from opening of the PAT hemiacetal moiety followed by retroaldolization and lactone hydrolysis (Fig. 4b). The high polarity of the suspected compound led us to try extraction with butan-2-one instead of diethylether. As expected, the GC peak at RI = 874 strongly increased.

To confirm our hypothesis, we synthesized 3-keto-5-hydroxypentanal (B in Fig. 5a) in diethylether from ethyl formate, 4-hydroxy-butan-2-one, and sodium ethoxide. The retention index and mass spectroscopy data corroborate our hypothesis (Fig. 5c to be compared with 4a). Worth stressing is the high propensity of 3-keto-5-hydroxypentanal to polymerization under acidic or basic conditions when its concentration is increased.

As the hemiacetal function has to be transformed into an aldehyde before retroaldolization and lactone hydrolysis, sulfites accelerate PAT degradation into 3-keto-5-hydroxypentanal by the formation of aldehyde-sulfitic adducts.

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