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要　　旨：近年、マイコトキシンに係る研究分野では、マイコトキシンの配糖体や誘導体の存在が注目されている。パツリンについては、配糖体の存在がこれまでに報告されていない。仮にパツリンに配糖体があった場合には、リスクを過小評価することになる。そこで本研究では、酵素反応によりパツリンの配糖体化について、dextransucraseを用い検討を行った。酵素反応液はLC-MSにより精密質量分析を行った。その結果、パツリンの配糖体化は確認できなかった。しかし、パツリンと同様の組成式をもつが、276 nmに吸収がない、パツリンとは異なる構造の化合物が検出された。この化合物はdextransucraseの作用で生成した化合物である可能性があり、dextransucraseはパツリンを構造変換させる可能性を示唆した。

Abstract：In recent years, the presence of mycotoxin glycosides and mycotoxin derivatives has emerged as a popular topic in the field of mycotoxin research. The presence of glycosides in patulin has not thus far been reported, however, if glycosides do exist in patulin, the risks may be underestimated. Given these background, this study examined whether patulin was glycosylated through an enzymatic reaction. Dextransucrase was used for the enzymatic reaction, and the enzymatic reaction solution was subjected to an accurate mass analysis by LC-MS to examine whether mass ions and the mass spectrum corresponding to patulin glucoside could be observed. In this study, the glycosylation of patulin was not be determined. However, a compound with the same formula, but different structure as patulin and showing no absorption at 276 nm was detected. This compound may have been generated in a reaction catalyzed by dextransucrase. Thus, this study indicated that dextransucrase may catalyze the structural transformation of patulin.

キーワード：マイコトキシン、グルコシド、マスクドマイコトキシン、パツリン、dextransucrase、精密質量分析

Keywords：mycotoxin, glucoside, masked mycotoxin, patulin, dextransucrase, accurate mass analysis

Introduction

Patulin, a mycotoxin produced by a variety of molds, particularly *Penicillium expansum* species, is toxic to animals. Administration of patulin at large doses can cause gastrointestinal, hepatic, and/or lung impairment, including congestion, bleeding, and necrosis[1-3]. The International Agency for Research on Cancer (IARC) has classified patulin as a group 3 carcinogen (not classifiable for its carcinogenicity to humans)[4]. Therefore, that hasn't been investigated in detail. The Joint FAO (Food and Agriculture Organization of the United Nations)/ WHO Expert Committee on Food Additives (JECFA) reported the provisional maximum tolerable daily intake (PMTDI) of patulin as 0.4 μg/kg bw/day[5]. Although there have been no human cases or epidemiological reports of patulin-poisoning, some cases of intoxication in bovines have been reported[6]. The toxicity of patulin *in vivo* is considered to result from its binding to the -SH groups of a physiological protein, which results in the denaturation of the protein and the subsequent onset of toxicity[7].

Damaged apples that cannot be sold as raw fruit are sometimes used as a raw material for fruit juice. Patulin-producing strains are known to enter and grow within damaged apples. Therefore, patulin may be found in the juice of apples or other fruit juices. As apple juice consumption per body weight is higher in infants and children than in adults, the health hazards of patulin to infants and children are a major concern. To prevent these hazards, the Japanese Ministry of Health, Labour and Welfare limited patulin content in apple juice to no more than 0.050 ppm in accordance with CODEX STAN 193-1995[8,9].

The reported cases of patulin content in apple juice include an investigation of 249 samples of domestic apple juice by the Ministry of Agriculture, Forestry, and Fisheries of Japan. This report stated that although patulin content was within the acceptable level in all samples, it exceeded quantification limit (0.010 ppm) in three samples, with the maximum patulin level being 0.021 ppm[10]. In addition to apples, patulin-contaminated grapes have also been reported. However, overall, reports of patulin detection are rare in Japan. Ohmichi Laboratory[11] analyzed 20 samples of apple juice and grape juice between 2013 and 2014, but did not detect patulin levels higher than 0.01 ppm in any sample.

In recent studies of mycotoxin assays, induced metabolites such as mycotoxin glucosides have attracted attention[12-15]. Mycotoxins are metabolized in the detoxification processes of plants. Mycotoxin glucosides, referred to as masked mycotoxins(or modified mycotoxins), are especially well known. Masked mycotoxins are undetectable by routine analytical techniques, which increase their chances of remaining undetected, thereby resulting in the underestimation of mycotoxin risk. As the ingested masked mycotoxins are decomposed by the endogenous enzymes of intestinal bacteria and the resultant "unmasked" mycotoxins exert toxicity, they are regarded as important factors in risk evaluation. Glucoconjugates of *Fusarium* toxins, such as deoxynivalenol glucoside, are known as naturally occurring masked mycotoxins. However, the identification of a patulin glucoside has never been reported. The existence of a patulin glucoside would mean that the risk from patulin has been underestimated.

Therefore, in this study, we investigated whether patulin is metabolized to glucoside by enzymatic reactions. Dextransucrase (sucrose: (1→6)-α-D-glucan 6-α-D-glucosyltransferase; EC. 2.4.1.5) is known as a glycosyltransferase supporting the construction of well-defined polysaccharides[16]. Dextransucrase (DS-ase) produced by *Streptococcus bovis* 148, isolated from bovine rumen, catalyzes the cleavage of sucrose and polymerization of the resultant glucose molecules via α-1,6 linkages[17]. This enzyme is also reported to produce riboflavin glucoside[18]. Considering these characteristics[16-18], we examined whether DS-ase could catalyze the glycosidation of patulin.

The enzymatic reaction solution was subjected to an accurate mass analysis by LC-MS to examine whether mass ions and mass spectral features corresponding to patulin glucoside could be observed.

Materials and Methods

Experimental materials

Patulin was purchased from Sigma-Aldrich (MO, USA).

Commercially available granulated sugar (MITSUI SUGAR) was used as the source of sucrose.

Streptococcus bovis 148, isolated from bovine rumen, was used as the test strain[17] and cultured in glucose-yeast extract-polypeptone (GYP) medium, with glucose serving as the carbon source. The cells were incubated for 20 h at 40°C under anaerobic conditions, by carbon dioxide aeration, and serial neutralization of the medium was performed using sterilized sodium bicarbonate powder. The culture solution was centrifuged (15,000×*g*, 15 min), and the obtained supernatant was dialyzed overnight against a jet of water. The resulting supernatant was used as the enzyme solution for this study. DS-ase activity was 18.10 units/mL when glucose was the substrate.

Instruments

Accurate mass spectra and chromatograms were obtained on an LTQ Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany) with a high resolution Orbitrap mass spectrometer (MS) connected to a high-performance lipid chromatography (HPLC) system comprising an Ultimate™ Rapid Separation LC system with a photodiode array (PDA) detector (Thermo Fisher Scientific).

General experimental procedures

DS-ase was added in the presence of sucrose at a 10-fold lower concentration than that of patulin in terms of the molar ratio, as shown below. The control for comparison consisted of the deactivated enzyme solution prepared by autoclave sterilization.

The composition of the enzyme reaction solution was:

32 mM Patulin (aqueous solution)	400 μl
3.2 mM Sucrose (aqueous solution)	400 μl
Enzyme solution	200 μl

The enzymatic reaction mixture was conducted at 40℃ for 24 h.

LC-MS analysis

Each reaction solution was subjected to LC-MS analysis. The LC-MS parameters for the operation of the electrospray interface (ESI) were optimized as follows: heater temperature 400℃, negative ionization mode, sheath gas 40 arbitrary units (au), aux gas 5 au, capillary temperature 230℃, I spray voltage 3.5 kV. Chromatographic separation was performed on an ACQUITY UPLC HSS C18 (100 × 2.11 mm I.D., 1.8 μm particle size) column (Waters, MA, USA) at 40℃ with flow rate of 0.3 ml/min. The carrier phrase comprised water/formic acid (99.9:0.1, v/v; solvent A) and acetonitrile/formic acid (99.9:0.1, v/v; solvent B). First, the solvent proportion was 5% B for 7 min, which was then increased to 90% B within 20 min, and held for 5 min at 90% B. Thereafter, the proportion of B was decreased to 5% within 0.1 min, and held at 5% for 4.9 min prior to the next sample injection. The LC-MS system was operated in full spectra acquisition mode, over a mass range of *m/z* 70-600, at an ultra high-resolution power of 100,000 (*m/z* 200). An accurate mass/high resolution full scan, all ion MS/MS spectrum acquisition with collision energy and PDA chromatography were simultaneously performed in a single run. Fragmentation was achieved in an optional collision induced dissociation (CID) cell, with collision energy of 35.0 eV. Each scan event was performed at negative polarity. Chromatograms obtained by PDA were extracted at a UV wavelength of 276 nm.

Results and Discussion

The chromatogram of the enzyme reaction solution is shown in Fig. 1. Patulin was detected at a retention time of 11.27 min. This was confirmed by the patulin-equivalent *m/z* 153 $[M-H]^-$ in the mass chromatogram (second panel in Fig. 1). The mass spectrum of patulin is shown in Fig. 2. The second panel of Fig. 2 shows the presence of ions with *m/z* values of 81, 109, 125, and 135 as fragments from cleaved patulin.

In the patulin-equivalent *m/z*: 153$[M-H]^-$ (the second panel of Fig. 1), a peak was seen at a retention time of 6.75 min. Essentially, a compound with the same composition-formula as patulin was eluted at a retention time of 6.75 min, which was different from the of patulin peak (11.27min) in the reaction mixture solution containing 400 μl of 32 mM patulin aqueous solution, 400 μl of 3.2mM sucrose aqueous solution, and 200 μl of DS-ase. The MS/MS spectrum of the compound, determined by CID, was similar to the cleavage pattern of patulin (Fig. 2 & 3).

Similar to Fig. 2, we obtained ions with *m/z* values of 81, 109, 125, and 135 in Fig. 3, thereby suggesting that the generated compound has a formula similar to that of patulin and eluted at a retention time of 6.75 min.

The fourth panel of Fig. 1 shows the PDA absorption at 276 nm. A strong peak derived from patulin was observed at 11.19 min (close to 11.27 min), but no peak was found at 6.75 min. The compound at 6.75 min was considered to have a structure that, for an unknown reason, lacked the relevant absorption.

This study showed that patulin may react with DS-ase to subsequently produce a compound similar to patulin in structure but lacking absorption at 276 nm.

In the mass chromatogram of the patulin glucoside-equivalent *m/z* 315.0690–315.0754 $[M-H]^-$, a peak was not found at the aforementioned retention time of 6.75 min (third panel of Fig. 1). In addition, although we could see certain strong peaks in this mass chromatogram extracted by patulin glucoside-equivalent *m/z* (third panel of Fig. 1), each MS/MS spectrum of detected compounds at patulin glucoside-equivalent *m/z* was not corresponded with the cleavage pattern of patulin. The results of this accurate mass analysis did not show the production of patulin glucoside in the enzyme reaction solution. Therefore, the generated compound was not a glucoside. The glycosylation of patulin was not be determined in this study. In addition, compared with the deactivated enzyme solution used as a control, peaks that were suggestive of the possible production of patulin glucoside were not identified.

This study demonstrated the possible conversion of patulin to an unknown compound that resembled patulin but lacked absorption at 276 nm. However there is a report that *Lactobacillus plantarum* can convert patulin to E-/Z-ascladiol or hydroascladiol through ring-opening[19]. Patulin biosynthesis involves a series of condensation and redox reactions, many, if not all, of which are enzyme catalyzed[20]. Structural changes in the presence of DS-ase may occur depending on the reaction conditions.

Although patulin glucoside has not been reported, there was a report of patulin linked amino acids or protein in cloudy apple juice and the compounds were named bound patulins[21]. Therefore, we suggest the consideration of a decontamination strategy for patulin or bound patulin using enzymes such as DS-ase.

In this study, using accurate mass analysis, we first examined the possible production of patulin. However, its presence was elucidated as an absorption peak at 276 nm was not detected by PDA. This was analytically significant because erroneous qualitative judgment can be overruled by accurate mass analysis combined with PDA detection. In this study, we were able to establish the importance of complete analysis using multiple detectors such as MS and PDA. The complementary use of these technologies should be incorporated into routine analysis.

It should be noted that this generated compound (retention time : 6.75 min) could not be reproduced; thus, it is possible that this may have been a contaminant in the detection. This aspect is still under examination.

In addition, it is important to note that in this study, it was shown that a false-positive peak for patulin glucoside or patulin itself can be identified through the combined use of mass chromatogram, MS/MS spectrum or chromatogram from the PDA data. The results of this study are indication of the importance of combinatorial

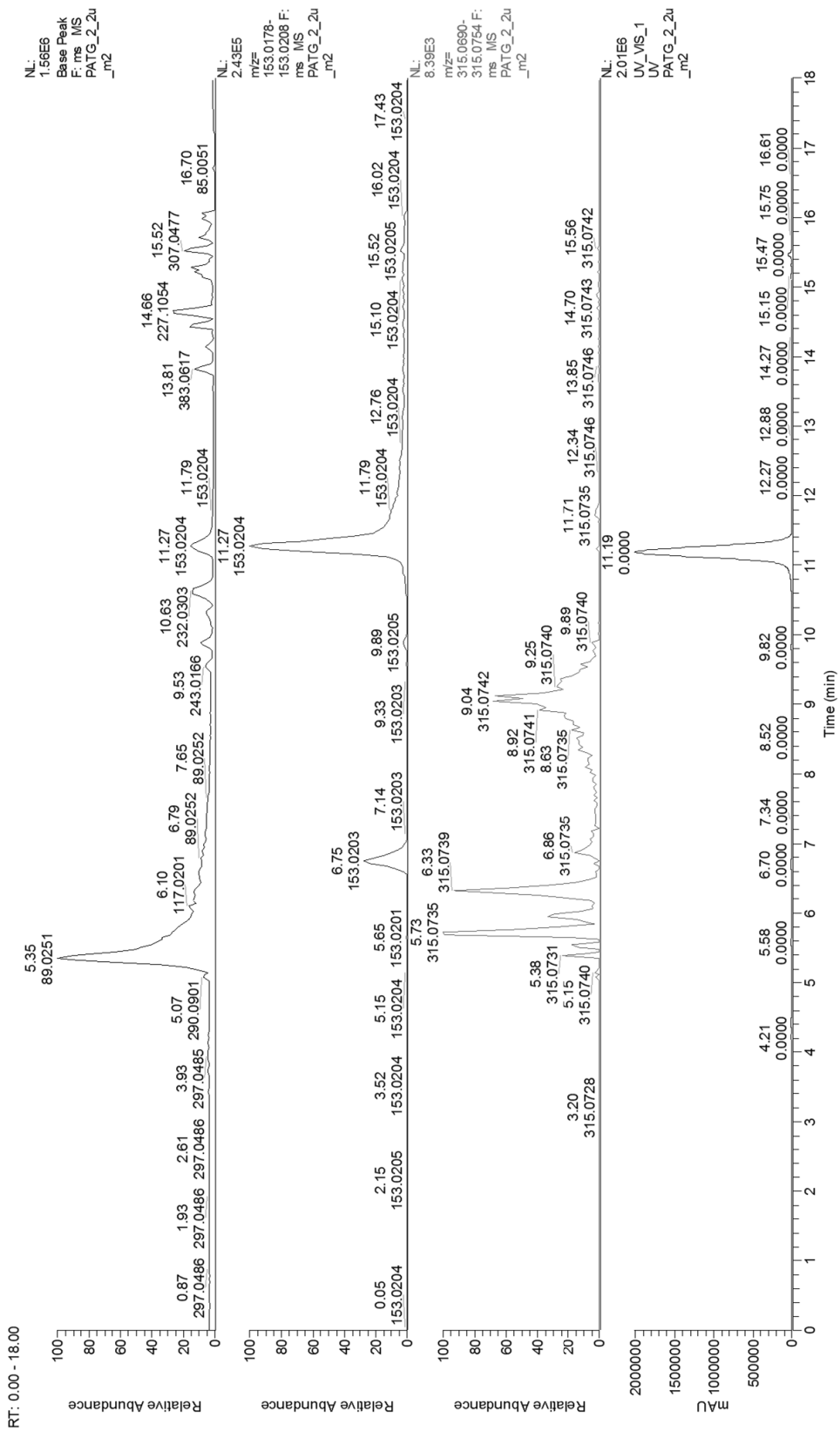

Fig. 1. Chromatogram of enzyme reaction solution
First panel: Total ion current chromatogram. Second panel: mass chromatogram at *m/z*: 153.0178-153.0208 (Patulin-equivalentmass).
Third panel: mass chromatogram at *m/z*: 315.0880-315.0754 (patulin glucoside-equivalent mass).
Fourth panel: Chromatogram extracted from the PDA data at 276 nm.

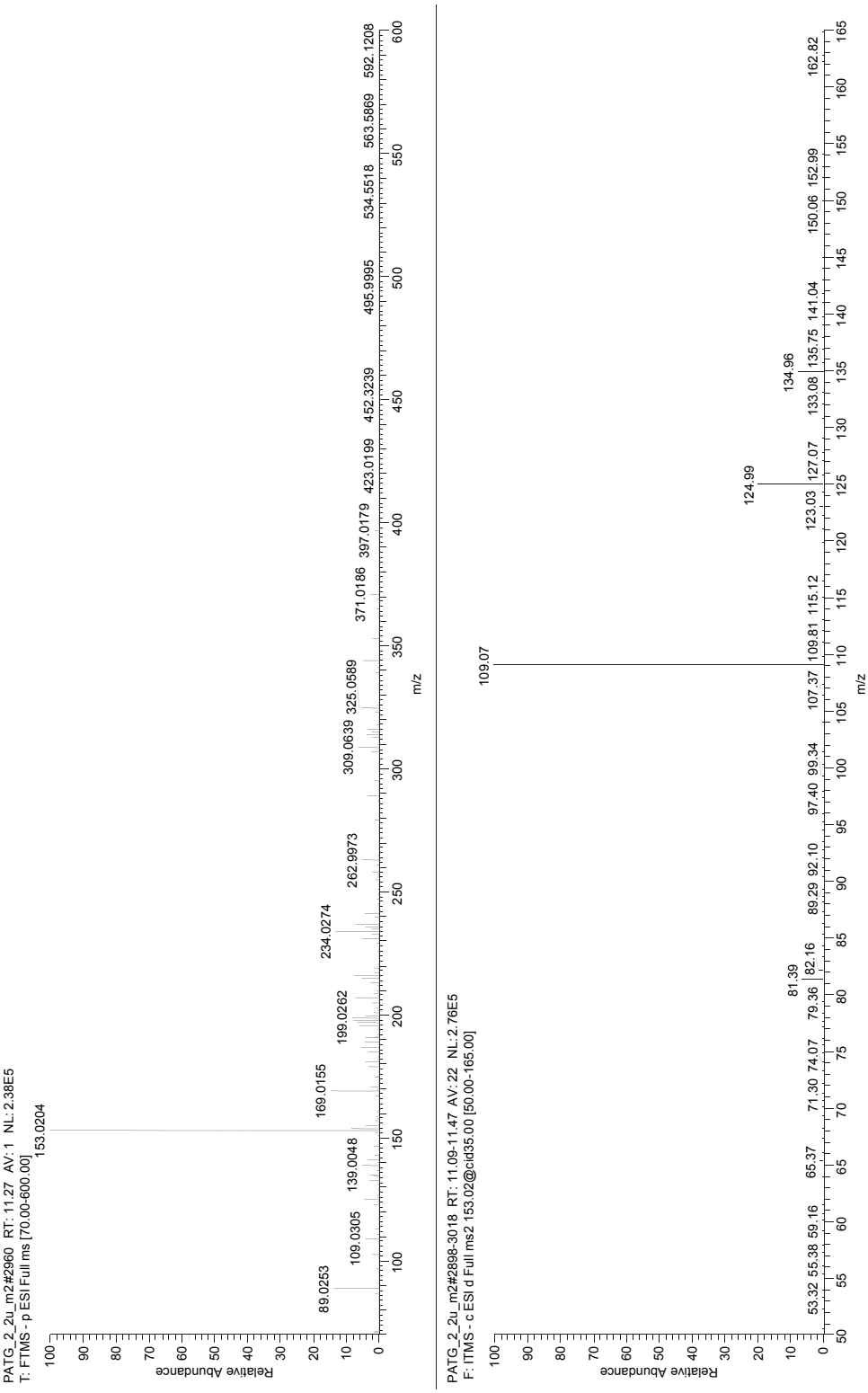

Fig. 2.　Mass spectrum obtained at 11.27 min
First panel: full scan results. Second panel: MS/MS spectrum of precursor ion *m/z* : 153 with collision energy.

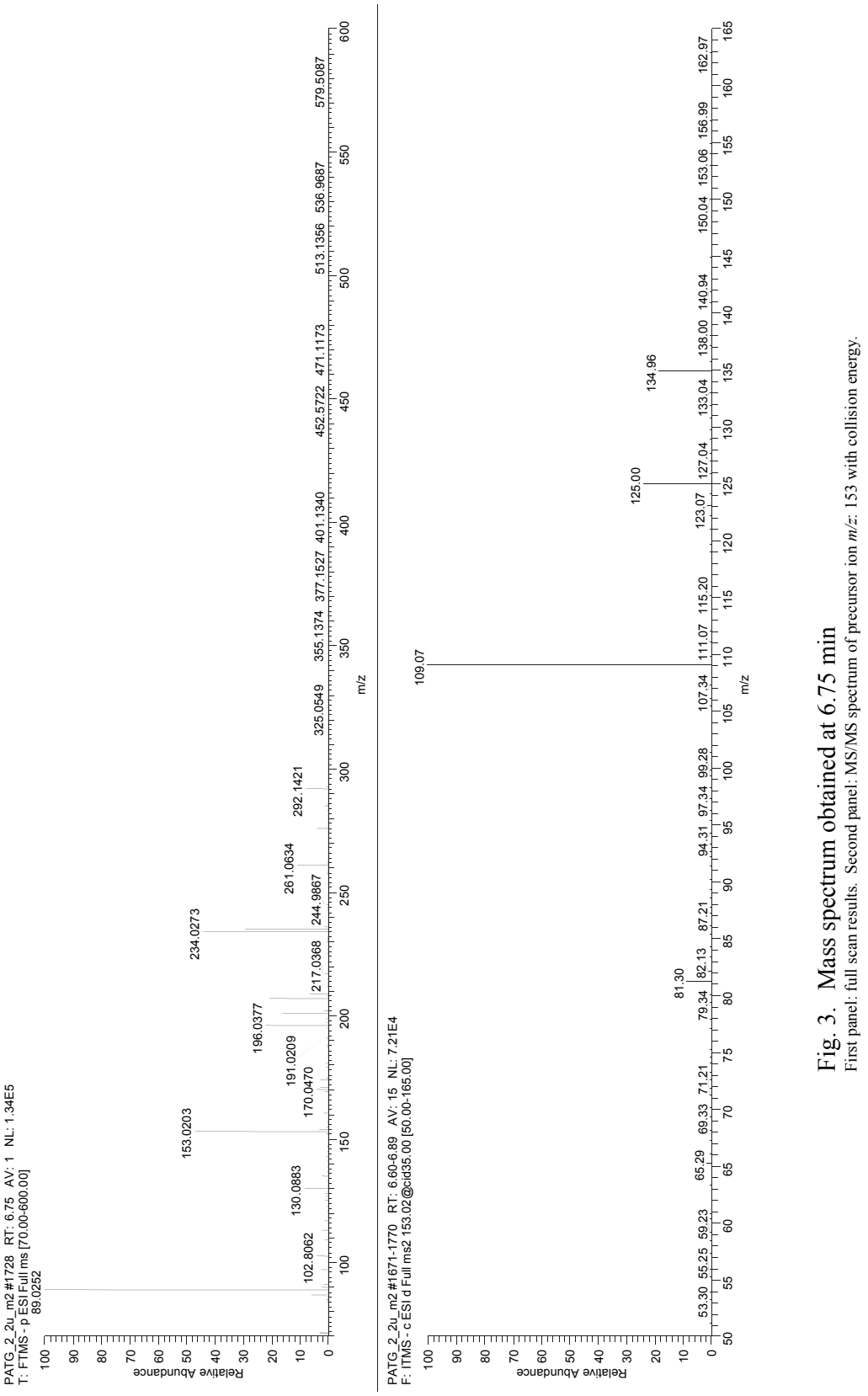

Fig. 3. Mass spectrum obtained at 6.75 min
First panel: full scan results. Second panel: MS/MS spectrum of precursor ion *m/z*: 153 with collision energy.

analytical approaches.

Conclusion

A compound with structural resemblance to patulin, but without absorption at 276 nm, was produced by the reaction of patulin with DS-ase. This finding shows the possible conversion of patulin to an unknown compound that resembles patulin. Naturally occurring patulin glucoside has never been reported. The production of patulin glucoside through enzymatic reactions was not successful. This study also suggests the importance of complete analysis using a combination of mass chromatogram, MS/MS spectrum or chromatogram from the PDA data.

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