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Original Research Article

Structural Identification of *Lentinus edodes* Cellulose Derivative that Inhibits Aflatoxin Production by *Aspergillus flavus*

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Abstract

Purpose: *To identify the structures of cellulose-extract derivative (CED) formed by heating Lentinus edodes cellulose in water surroundings that can efficiently inhibit aflatoxin production by Aspergillus flavus.*
Methods: *CED was purified on Sepharose CL-6B columns, and then structurally characterized using amino acid analyzer, gas chromatography, Fourier transform infrared spectroscopy, and polyacrylamide gel electrophoresis.*
Results: *CED completely inhibited aflatoxin AFB1 production by A. flavus at concentrations ≥ 100 μg/mL. Chemical analysis indicated that CED contained 82 % carbohydrate and 18 % protein and has a molecular weight of approximately 24 kDa. Monosaccharide component analysis indicates that glucose was the predominant monosaccharide of CED. Analysis by Smith degradation and enzymatic hydrolysis indicate that there were only (1, 4)-glycosidic linkages in the CED polysaccharide chains. The protein backbone of CED contained 15 kinds of amino acid with higher levels of glutamic acid, aspartic acid, leucine and alanine.*
Conclusion: *CED was identified as a complex of peptide and polysaccharide structures possessing β-(1, 4)-glucan backbones, and it provides a theoretical basis for developing polysaccharide preparations to control aflatoxin contamination with medical and food science applications.*

Keywords: *Aflatoxin B_1, Lentinus edodes, Aspergillus flavus, Cellulose derivative, Structure identification, Amino acid*

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INTRODUCTION

Aspergillus flavus occurs as a saprophyte in soils worldwide and causes diseases in several important crops, such as maize, peanut, and cottonseed, before and after harvest [1]. The greater concern is that *A. flavus* can produce aflatoxin B_1 (AFB_1), which has been implicated as a causal agent of liver cancer in humans and other animals [2]. Control of aflatoxin contamination has been a worldwide problem. Extensive strategies have been developed and improved to prevent fungal colonization and aflatoxin formation in crops.

Glucans from mushrooms, pharmacologically classified as biological response modifiers and most immune modulator, have been found to

attenuate aflatoxicosis [3,4]. Reverberi *et al* [5] reported that lyophilized filtrates from *Lentinula edodes* inhibit aflatoxin production, and this inhibition is correlated with filtrate β-glucan content. The next year, another study from this laboratory showed a close relationship between β-glucans amount in *Trametes versicolor* extracts and aflatoxin inhibition ability and demonstrated that β-glucans could be involved in aflatoxin inhibition [6].

In this study, we discovered that hot water-separated cellulose-extract derivative (CED) from *L. edodes* fruiting bodies can efficiently inhibit aflatoxin production by *A. flavus*. Our further studies were, therefore, carried out to characterize the structure of this CED.

EXPERIMENTAL

Materials and chemicals

Standard grade AFB1, arabinose, xylose, glucose, and glucuronic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA; purity ≥ 95 % by HPLC). Solvents and other chemicals were of analytical or HPLC grade (purchased from Sangon Biotech, Shanghai, China). *L. edodes* purchased from a local market in Xinxiang, China. *A. flavus* CGMCC 3.2890 was purchased from China General Microbiological Culture Collection Center (CGMCC Beijing, China).

Cellulose extract derivative (CED) preparation

Cellulose was extracted from *L. edodes* fruit based on a method described by Yan *et al* [7] with some modifications. Fresh *L. edodes* fruits were cleaned with water and then dried at 60 °C. The dried fruits were crushed into powder with a steel grinder (HX-100 grinder, Tongqi Instrument Co., Ltd, Hangzhou, China) and then boiled in distilled water for 4 h. The mixture was filtered through a linen cloth, and the residue was washed with distilled water and ethanol to remove organic acids and saccharides. The precipitate was then washed with distilled water several times through Whatman No.1 filter paper and the dried cellulose was used for CED production.

Ten grams of powdered cellulose extract were re-suspended in 100 mL of distilled water for 15 min at 125 °C, and then filtered through Whatman no. 1 filter paper. The filtrate was concentrated to 25 mL using a rotary evaporator at 50 °C, applied to a Sepharose CL-6B column (1.6 × 100 cm), and eluted with water at a flow rate of 0.5 mL/min. The phenol-sulfuric acid method was used to monitor the collected fractions. The void volume was determined by blue dextran, and standard T-dextran was used to calibrate the column to determine fraction molecular weights [8].

Aflatoxin production and quantification

Sabouraud's medium (peptone 1 g, glucose 4 g, water 100 mL) was used to culture *A. flavus* for aflatoxin production. CED was evaluated for aflatoxin inhibition by addition to media (10–1000 μg/mL) before inoculating with *A. flavus* CGMCC 3.2890 spores (≈105 mL^{-1}). The inoculums were incubated in flasks at 30 °C, with rotational shaking at 120 rpm for 72 h. Aflatoxin B_1 in cultures was determined and compared to control cultures (without tested fractions). Three replicates were performed for each concentration, and the experiment was repeated three times.

Aflatoxin B_1 was extracted from *A. flavus*-conditioned medium with three volumes of chloroform. Extracts were dried with a nitrogen blowing instrument, dissolved in methanol, and then filtrated through a 0.22-μm microporous membrane for subsequent HPLC analysis. Samples were injected into HPLC system (Model 1100; Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with an octadecylsilyl column (Cosmosil 5C18-AR column, 250 × 4.6 mm; Nacalai Tesque, Inc, Kyoto, Japan) kept at 22 °C The mobile phase was acetonitrile/methanol/ water (1:1:2, v/v/v) at a flow rate of 1 mL/min. The column eluent was passed through a variable wavelength detector operated at 365 nm and calculated against an Aflatoxin B_1 standard curve [9].

Compositional analysis

Total CED carbohydrate content was determined by the phenol-sulfuric acid colorimetric method using glucose as the standard. Protein content was quantified using the Bradford method with bovine serum albumin as standard [10,11]. Amino acid composition was determined using an amino acid analyzer following the method outlined in Beckman/Spinco instruction manual.
Monosaccharide analysis was performed as previously described [12]. Ten mg of CEDs were hydrolyzed with 2 M trifluoroacetic acid (TFA) at 120 °C for 3 h, and the hydrolyzed products were co-evaporated with alcohol to remove residual TFA until the pH was ≈ 7. The hydrolyzed products were reduced with $NaBH_4$ at room temperature for 1 h and neutralized with 50 % acetic acid. After desalting with cation-exchange

resin, the hydrolysis products were filtered through quantitative filter paper and neutralized by methanol addition and evaporation, and the reaction solution was mixed with 1 mL n-propylamine and anhydrous pyridine for 30 min at 55 °C. Finally, the dried sample was mixed into 0.5 mL anhydrous pyridine and acetic anhydride for 1 h at 90 °C.

The derived acetic esters were analyzed by GC (TRACE GC 2000, Thermo Finnigan, LLC, San Jose, CA, USA), using the following conditions: H_2:16 mL/min; air: 150 mL/min; N_2:20 mL/min; injection temperature: 230 °C; detector temperature: 230 °C; column temperature programmed from 130 to 180 °C at 5 °C/min, holding for 2 min at 180 °C, then increasing to 220 °C at 5 °C/min and finally holding for 3 min at 220 °C. Sugar identification was done by comparison with reference sugars (arabinose, xylose, glucose and glucuronic acid).

The infrared spectrum of CED was determined using a FT-IR spectrometer equipped with OPUS 3.1 software (Bruker Optics, Ettlingen, Germany). Five milligrams of the derivatives were ground with KBr powder and then pressed into pellets for transformation infrared spectrum measurement in the range of 400-4000 cm^{-1} [13].

Periodate oxidation and Smith degradation

Twenty-five mg of CED was dissolved in 12.5 mL of distilled water mixed with 12.5 mL of 30 mM $NaIO_4$, and the mixture was kept in the dark at 4 °C. Aliquots (0.1 mL) were withdrawn from the mixture at 3 – 6 h intervals, diluted to 25 mL, and read in a spectrophotometer at 223 nm. The reaction was completed when absorbance stopped decreasing, and the excess $NaIO_4$ was decomposed by ethylene glycol addition (0.2 mL). Consumption of $NaIO_4$ was measured by a spectrophotometric method and the production of formic acid was determined by titration with 0.00458 M NaOH aqueous solution.

The reaction mixture was dialyzed in moving tap-water (24 h) and distilled water (24 h), and the retained material was concentrated and reduced with $NaBH_4$, neutralized with 50 % acetic acid, and then dialyzed. The content inside the dialysis bag was lyophilized and analyzed by one-third of the GC content inside the dialysis bag was lyophilized and analyzed by GC. The rest of the fraction was hydrolyzed by adding an equal volume of 1 M H_2SO_4 for 40 h at 25 °C, neutralized to pH 6.0 with $BaCO_3$, and filtered for analysis of Smith degradation. The filtrate was dialyzed, and the bag contents were desiccated for GC analysis; the content inside was diluted with ethanol, and the supernatant and precipitates were also dried out for GC analysis after centrifugation [10].

Enzymatic hydrolysis and electrophoretic analysis

CED (200 mg) was treated with proteinase K (600 U, Tritirachium album, Sigma) in the phosphate buffer, pH 7.5 (10 ml) at 37 °C for 3 h. CED (200 mg) was treated with cellulase (400 U, Trichoderma Viride, Baoman Biological Technology Co, Ltd, Shanghai, China) in the phosphate buffer, pH 7.5 (10 ml) at 63 °C for 3 h. Then digests were analyzed by polyacrylamide gel electrophoresis (1-mm-thick containing 7.5 % polyacrylamide, 0.09 M Tris, 0.08 M boric acid, and 2.6 mM EDTA). The sample buffer was Tris-boric acid-EDTA, pH 8.3, containing 0.2 M Tris-base, 0.2 M boric acid, 2.0 M sucrose, and 20 mM EDTA. One volume of sample buffer was mixed with one volume of the CED sample. Gels were run for 1 h at a 200-V in double-diluted sample buffer containing no sucrose. Polysaccharides were visualized by staining with silver [14].

Statistical analysis

Data were expressed as the mean of three replicate determinations ± standard deviation (SD). Statistical comparisons were performed by Student's test, using SPSS v.17.0 software (IBM/SPSS, Chicago, IL, USA). $P < 0.05$ was considered to be indicative of statistically significant difference.

RESULTS

Derivative inhibits aflatoxin production

By gel filtration, a single peak (molecular weight ≈ 24 kDa) formed on the saccharide curve based on fraction analysis, which coincided with the peak on the fraction protein content curve. This observation indicates that CED was a complex of saccharides and peptides. Further analysis showed that CED concentration-dependently inhibited AFB_1 production by *A. flavus*, with an IC_{50} of < 10 µg/mL. Concentrations ≥ 100 µg/mL completely inhibited AFB_1 production by *A. flavus* ($p < 0.05$) (Fig. 1).

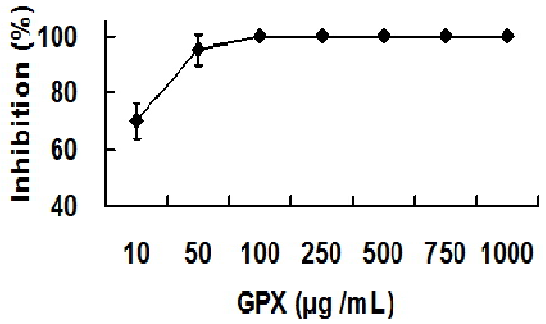

Figure 1: CED concentration-dependently inhibits AFB_1 production by *A. flavus* CEDs ($p < 0.05$ for all tested concentrations)

Chemical and structural characteristics of CED

CED contained approximately 82 % carbohydrate and 18 % protein, as respectively determined by the phenol-sulfuric and Bradford methods. The protein backbones of amino acid analysis indicated that CED is rich in glutamic acid, aspartic acid, leucine, and alanine (Table 1). By GC analysis of hydrolyzed CED, comparison with standard saccharides (arabinose, xylose, glucose, and glucuronic acid) indicated that CED contained only glucose (Fig 2).

The Fourier transform infrared (FTIR) spectrum of CED displayed a broad intense characteristic peak at ≈ 3389 cm^{-1} for the hydroxyl group, and a weak C-H stretching band at 2934 cm^{-1} (Fig 3). The peak at 2346 cm^{-1} also indicates C-H bonds.

Table 1: Amino acid composition of CED protein components

Amino acid	Content (%)
Glutamic acid	3.114
Aspartic acid	2.527
Leucine	1.603
Alanine	1.297
Threonine	1.284
Serine	1.238
Valine	1.075
Isoleucine	0.923
Lysine	0.839
Arginine	0.831
Phenylalanine	0.78
Tyrosine	0.73
Proline	0.438
Methionine	0.325
Cystine	0.097
Total	17.101

The relatively strong absorption peak at ≈ 1634 cm^{-1} and the weaker peak at 1250 cm^{-1} also indicated the characteristic IR absorption of a polysaccharide. A wider symmetrical peak near 1403 cm^{-1} was an indication of carboxyl groups [15].

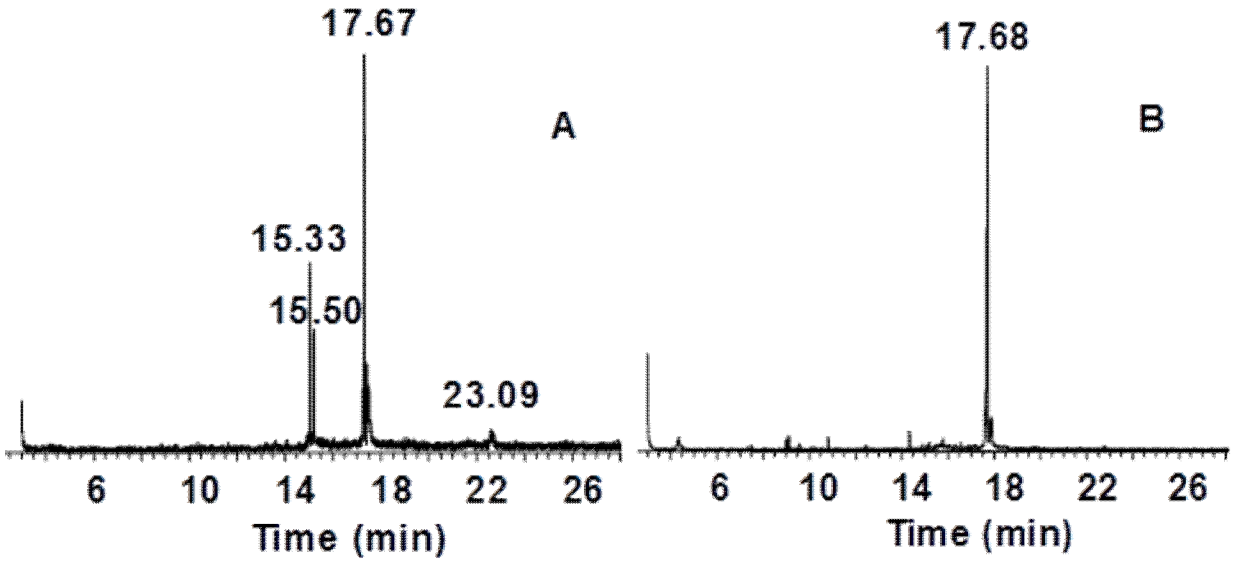

Figure 2: A = Gas chromatogram (GC) of CED monosaccharide standard (elution time for monosaccharides: 15.33 min, arabinose; 15.50 min, xylose; 17.67 min, glucose; 23.09 min, glucuronic acid); and **B =** GC profile of hydrolyzed CED polysaccharide hydrolyzate

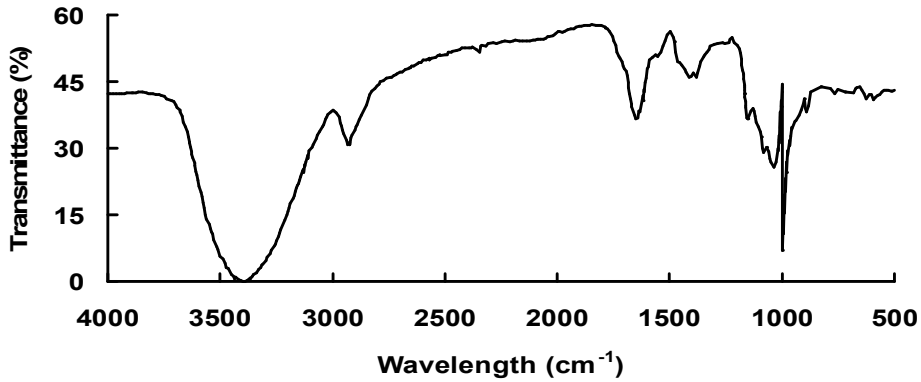

Figure 3: Fourier transforms infrared spectrometery of CEDs

Characteristically, the three bands near 1075 cm^{-1} suggested the presence of a pyranose ring of the glucosyl residue in the component polysaccharide. Furthermore, the characteristic absorptions observed at 892 cm^{-1} in the IR spectra indicated β-configurations existing in CED [10].

Oxidation of CED with 30 mM sodium periodate at 4 °C was completed in 10 days and the consumed periodate was 0.53 mmol. Based on molecular weight calculations, CEDs contain approximately 170 glycosidic bonds. No formic acid was generated after CED periodate oxidation, which indicated that hexapyranose with 1→3 or 1→6 glycosyl linkages were not present. GC analysis showed the presence of only erythritol, which indicated that hexapyranose with only 1→4 glycosyl linkages, existed in CED [16].

Figure 4: Electrophoretic analysis of intact CED (lane 3) and enzymatic degradation products generated by protease K and cellulase treatment (lanes 1 and 2, respectively), showing complete CED hydrolysis with either enzyme treatment

Protease K is a broad-spectrum serine protease. Cellulase catalyzes the specific hydrolysis of endo-1, 4-β-D-glycosidic linkages in cellulose. As shown in Figure 4, CEDs were completely hydrolyzed by protease K or cellulase, which further confirmed that the CED was β-(1, 4)-glucan-peptide complex.

DISCUSSION

The filtrates of some mushroom extracts inhibiting aflatoxin production are promising agents for aflatoxin control strategies because of their low cost and food grade quality. Some β-glucans such as lentinan in the filtrates were thought to be probably the valid constituent against aflatoxin biosynthesis [5]. However, previous studies indicate that some β-glucans have very low inhibitory activity against aflatoxin production, and that lentinan only exhibited limited inhibitory activity within a narrow concentration range. This study revealed that the CED is probably responsible for the inhibition of aflatoxin production by mushroom extracts.

CED is highly water-soluble even at room temperature, and is formed only by treating starting plant materials at high temperature (i.e., > 80 °C). These results indicate that CED is a product of chemical modification of native plant compounds. We identified CED as a complex of peptide with polysaccharides possessing β-(1, 4)-glucan backbone. We speculate that the polysaccharides exist in short chains (maybe like antennae) because long chains of β-(1,4) glucans have poor water-solubility.

Several studies have revealed that glucan can attenuate aflatoxin B_1 damage through multiple ways. β-D-glucans (1, 3 and 1, 6) isolated from *Saccharomyces cerevisiae* could adsorb aflatoxin B_1, reduce its bioavailability in the digestive tract, and protect animals against its adverse effects [17]. AFB_1 can enhance the ROS formation, cause oxidative DNA damage, and induce hepatocarcinogenesis [18]. β-(1, 3)-D-glucan could protect the hepatocytes against cell lysis and all oxidative stress cytotoxicity endpoints caused by ROS formation [19]. Our

result confirmed a new way that β-(1, 4)-glucan complex could inhibit the aflatoxin production. Whether the CED has other biological activities remains to be further investigated, and after all the lentinan from the mushroom have been developed as medicines to cure cancer.

There exists good correlation between increased cellular lipoperoxidation activity and the fungal antioxidant defense system that forms aflatoxins [20]. We hypothesize that cell-surface receptors in *A. flavus* bind to CED polysaccharides and shift the oxidant/antioxidant balance, leading to reduced aflatoxin production. The G-protein-coupled receptor tied to FadA mediates carbon source sensing, and its binding elicits signals that control aflatoxin biosynthesis [21]. Whether similar GPCRs can sense CED remains to be confirmed, and the mechanism of aflatoxin inhibition by CED is still under investigation.

CONCLUSION

Our study revealed that the water-soluble CED can be formed at increased temperatures, and that ≥ 100 μg/mL of these derivatives can completely inhibit aflatoxin production by *A. flavus*. These CEDs are identified as complexes of peptides and polysaccharides possessing β-(1, 4)-glucan backbones. CED provides a theoretical basis for developing novel polysaccharide preparations to control aflatoxin contamination, which would be useful in medicine and food science.

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