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Identification of a species-specific protein of toxigenic *Fusarium verticillioides*

Identificação de uma proteína espécie-específica de *Fusarium verticillioides* toxigênico

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Highlights

p67 has potential to be used as species-specific antigen of *F. verticillioides.*
This protein showed amylase activity and did not show protease activity.
UPLC-MS/MS analysis identified p67 as putative *F. verticillioides* glucoamylase GMY2.

Abstract

Fusarium verticillioides is a primary corn pathogen and the main producer of fumonisins, a group of toxic secondary metabolites that can cause human and animal health hazards. In humans, epidemiological studies have associated fumonisins with esophageal and liver cancer and have been classified by the International Agency for Research on Cancer – IARC in group 2B (possibly carcinogenic to humans). The development of a rapid, sensitive and specific method to detect this fungus is essential and immunological detection is a promising approach. The discovery, identification and characterization of species-specific antigens are important for the development of a specific immunological technique. Therefore, the objective of this study was to identify the 67 kDa protein (p67) of *F. verticillioides* exoantigen which shows high potential to be a species-specific antigen. p67 was identified by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) and by in-gel analysis of the enzymatic activity (amylase and protease) using polyacrylamide gel co-polymerized with starch, gelatin, and bovine serum albumin. The analysis of in-gel trypsin digested p67 by mass spectrometry revealed two possible

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proteins: the protein related to aminopeptidase Y precursor of *F. fujikuroi* and the putative glucoamylase GMY2 of *F. verticillioides.* In order to confirm which of these proteins the p67 is, both the enzymatic activities were evaluated and showed that this protein presents an amylase activity indicating that p67 is the putative glucoamylase GMY2 of *F. verticillioides.*
Key words: Corn. Food-borne fungi. Fumonisin. Immunodetection. Mycotoxin. Toxigenic fungi.

Resumo

Fusarium verticillioides é um patógeno primário do milho e o principal produtor de fumonisinas, um grupo de metabólitos secundários tóxicos que podem causar danos tanto à saúde humana como animal. Em seres humanos, estudos epidemiológicos indicam a sua associação com o câncer esofágico e hepático, sendo classificadas pela Agência Internacional de Pesquisa sobre o Câncer no grupo 2B (possivelmente carcinogênico para seres humanos). O desenvolvimento de um método rápido, sensível e específico para detectar este fungo é essencial e a detecção imunológica é uma abordagem promissora. A descoberta, identificação e caracterização de antígenos espécie-específicos são importantes para o desenvolvimento de um método imunológico específico. Portanto, o objetivo deste estudo foi identificar a proteína de 67 kDa (p67) obtida do exoantigeno de *F. verticillioides* que possui um grande potencial em ser um antígeno espécie-específico. p67 foi identificada por Cromatografia Líquida de Ultra Eficiência acoplada à Espectrometria de Massas (UPLC-MS/MS) e pela avaliação da sua atividade enzimática (amilase e protease) usando gel de poliacrilamida co-polimerizado com amido, gelatina, e soro albumina bovina. A análise da p67 digerida com tripsina por espectrometria de massas revelou duas proteínas possíveis: a proteína relacionada ao precursor da aminopeptidase Y de *F. fujikuroi* e a suposta glucoamilase GMY2 de *F. verticillioides.* Para confirmar qual dessas proteínas é a p67, ambas as atividades enzimáticas foram avaliadas e mostraram que esta proteína apresenta atividade amilase indicando que p67 é a suposta glucoamilase GMY^2 de *F. verticillioides.*
Palavras-chave: Fumonisinas. Fungos de origem alimentar. Fungos toxigênicos. Imunodetecção. Micotoxina. Milho.

Introduction

Fusarium verticillioides (Sacc.) Nirenberg (synonym, *F. moniliforme* (J.) Sheldon; teleomorph, *Gibberella moniliformis* (synonym *G. fujikuroi* mating population A) is an important corn pathogen which causes asymptomatic infection as well as severe rotting in all parts of the plant (Council for Agricultural Sciences and Technology [CAST], 2003). In addition to damaging the plants, *F. verticillioides* produces fumonisins, a group of mycotoxins which are associated with neural tube defects (Marshall et al., 2017) and esophageal and liver cancer in humans (Chuturgoon et al., 2014; Wang et al., 2014). The International Agency for Research on Cancer (International Agency for Research on Cancer [IARC], 2002) has classified them as possibly carcinogenic to humans (Group 2B). In animals, these mycotoxins are associated with leukoencephalomalacia in equines (Vendruscolo et al., 2016), pulmonary edema in swine (Pósa et al., 2016) and have hepatotoxic and hepatocarcinogenic effect in rats (Szabó et al., 2016).

Natural occurrence of toxigenic *Fusarium* species can indicate the presence of fumonisins in commodities (CAST, 2003). The occurrence of toxin-producing moulds in food and feed commodities is of concern and therefore, suitable methods for monitoring toxigenic fungi are required to control and implement regulating strategies.

Culture in several media, microscopic examinations and chemical analyzes of chitin, ergosterol and secondary metabolites are the methods traditionally used to detect mold and mycotoxin contamination (Yeni et al., 2014).

In addition to presenting low specificity and sensitivity, these methods require long analysis times, with the exception of secondary metabolite identification by chromatography and mass spectrometry. Chromatographic methods provide high sensitivity and specificity, but they use toxic reagents, are laborious, and require extensive sample cleanup.

The enzyme-linked immunosorbent assay (ELISA) is an alternative method for *F. verticillioides* detection because it allows analysis of several samples in a single test, simple sample processing and shows high sensitivity and specificity. Moreover, ELISA can detect the presence of fungi in food even after heat treatment which enables the evaluation of contamination in processed foods. ELISA based on exoantigens, which are immunogenic macromolecules produced and released to the culture medium throughout the growth of the fungus, is broadly employed for pathogenic fungi identification and detection because most fungi produce species-specific exoantigens (Kaufman & Standard, 1987).

Taking into account the frequent corn contamination with *F. verticillioides,* specific methods need to be developed to detect this fungus to improve corn quality control. Most ELISAs developed to date to detect *Fusarium* species in food are genus specific (Iyer & Cousin, 2003; Meirelles et al., 2006; Park et al., 2003). Therefore, it is essential to identify and characterize species-specific antigens which show potential to differentiate F. verticillioides, the main fumonisin producer, from other Fusarium species.

Biazon et al. (2006) described a possible species-specific protein of *F. verticillioides* isolates with apparent molecular weight of 67 kDa. According to previous studies, the indirect competitive ELISA using antibodies specific to 67 kDa protein (p67) was able to detect *F. verticillioides* exoantigen in poultry feed samples (Omori et al., 2019). The concentration of *F. verticillioides* exoantigen determined by this method correlated positively ($p < 0.05$) with the fumonisin levels ($\rho = 0.76$), which reinforces the idea that p67 is specific for *F. verticillioides* (Omori et al., 2019).

Although the use of p67 could increase the specificity of the method, the purification of the protein from the exoantigen is laborious and shows low yield. Therefore, it is important to identify this protein to determine the gene responsible for its synthesis which would allow the production of a recombinant p67. Mass spectrometry (MS) has been widely used to identify several compounds of different organisms (Ho et al., 2002; Kondo et al., 2006).

Taking into account the need for specific and sensitive methods for *F. verticillioides* detection and the probable

species-specificity of p67, the aim of this study was to identify this protein for future development of an immunological method based on recombinant p67 to detect *F. verticillioides* in food.

Material and Methods

Fusarium verticillioides culture

F. verticillioides 97K isolate was provided by the Mycological Culture Collection of the Department of Food Science and Technology at the State University of Londrina, Paraná, Brazil, and was routinely grown on potato dextrose agar at 25 °C.

Obtention of F. verticillioides exoantigen

F. verticillioides 97K exoantigen was produced according to Meirelles et al. (2006). Briefly, 10^7 conidia mL^{-1} suspension from *F. verticillioides* 97K grown on potato dextrose agar for seven days was prepared in sterile 0.15 mol L^{-1} phosphate buffer saline (PBS) containing 0.1% Tween 80 and aliquots of 1 mL were transferred to 250 mL brain heart infusion broth. The cultures were incubated at 28 °C, 150 rpm, for 14 days and were inactivated with 0.02% thimerosal at 4 °C for 24 h, followed by vacuum filtration and centrifugation at 4,500 x g at 4 °C for 20 min. The supernatant containing the exoantigen was dialyzed at 4 °C for 24 h with distilled water and PBS in dialysis tubes with exclusion limit of 12 – 16 kDa. It was then lyophilized and suspended in distilled water. Protein concentration was determined according to Bradford (1976) using bovine serum albumin (BSA) (Sigma, Saint Louis, USA) as standard.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

p67 was separated from other proteins of the *F. verticillioides* 97K exoantigen by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). Briefly, *F. verticillioides* 97K exoantigen was mixed with sample buffer (80 mmol L^{-1} Tris; 6% v/v SDS; 46% v/v glycerol; 7.7% v/v β-mercaptoethanol; 0.0125% v/v bromophenol blue), boiled for 5 min and 15 µL of the mixture and 10 µL of the molecular weight standard from 10 to 200 kDa (Invitrogen, Carlsbad, USA) were applied in the channels of the gel (5% stacking gel and 10% resolving gel). The electrophoresis was carried out at 100 V until the dye reached the end of the gel. The gel was then stained with Coomassie Brilliant Blue (Biorad, Hercules, USA). BSA was submitted to the same process to be used as control in Mass spectrometry (MS).

The p67 concentration was estimated by the software Image J (Research Service Branch, National Institute of Health, USA).

In-gel digestion and peptide extraction

The digestion of p67 and the extraction of its peptides from the gel were performed according to Shevchenko et al. (2006). Briefly, after electrophoresis and gel staining with Coomassie, the bands of p67 were detached from the gel, cut into small cubes, transferred to a microtube and destained with 100 mmol L^{-1} ammonium bicarbonate (Sigma, Saint Louis, USA) in 50% acetonitrile (ACN) (Sigma, Saint Louis, USA). The gel containing the target protein was then dehydrated with pure ACN, vacuum-dried and treated with trypsin solution (Sigma,

Saint Louis, USA) at 20 ng μL^{-1} overnight. The extraction buffer containing 5% v/v trifluoroacetic acid (TFA) (Sigma, Saint Louis, USA) in 67% v/v ACN was then added to the microtube followed by incubation at 37 °C for 1 h under gentle agitation. The supernatant containing the peptides was then transferred to a new microtube, vacuum-dried and stored at -20 °C until the analysis by UPLC-MS/MS.

The BSA and a fragment of the gel without any protein were submitted to the same procedure and were used as controls in MS.

Mass spectrometry analysis

Electrospray ionization tandem mass spectrometry (ESI-MS/MS) using a quadrupole time-of-flight (Q-TOF) linked to ultra-performance liquid chromatography (UPLC) (Bruker Corporation, Massachusetts, USA) was used to analyze peptides generated from the trypsin digestion in gel. The dried extracts of p67, BSA and gel without any protein were resuspended in 0.1% TFA. The system was equipped with a 150 x 2.0 mm C18, 3µm column (Phenomenex, California, USA) and the solvents used were as follows: 0.03% v/v TFA (solvent A); 90% ACN v/v with 0.03% v/v TFA (solvent B). The elution gradient used was 0–30 min, 2–60% B; 30–36 min, 60 – 90% B; 36–45 min, 90–2% B at 40 °C with a 200 $\mu L\ min^{-1}$ flow rate and 10 µL injection volume. A 1.3 kV ESI voltage in positive ion mode, 3 bar nebulizing pressure and 9 $L\ min^{-1}$ drying gas flow were applied for ionization using nitrogen.

The data was processed using Data Analysis (Bruker, California, USA) and the protein was identified using the Mascot software (Matrix Science, London, England) with the following parameters: National Center for Biotechnology Information (NCBI) for database, all entries for taxonomy, trypsin for enzyme, one missed cleavage allowed, peptide mass tolerance of ± 1.2 Da, fragment mass tolerance of ± 0.6 Da, non-fixed modification, non-variable modification, ion score cut-off of 20 and $p < 0.05$ for significance threshold.

In-gel analysis of the p67 enzymatic activity

Amylase activity

F. verticillioides p67 exoantigen amylase activity was determined according to Martínez et al. (2000) with some modifications. Briefly, *F. verticillioides* 97K exoantigen was mixed with sample buffer (0.125 mol L^{-1} Tris; 20% v/v glycerol; 0.04% v/v bromophenol blue) and was submitted to the SDS-PAGE in a 10% resolving gel containing 0.25% v/v copolymerized starch. The gel was subjected to 100 V at 4 °C until the dye reached the end of the gel. After electrophoresis, the gel was washed for 1 h with 2.5% Triton X-100 at room temperature and then was incubated for 3 h in 0.1 mol L^{-1} Tris-HCl pH 7.6 containing 2 mmol L^{-1} CaCl2 at 39 °C. After this period, the gel was washed with distilled water, the bands were fixed with 12% w/v trichloroacetic acid, and the gel was stained with lugol solution (6.7 mg mL^{-1} KI and 3.3 mg mL^{-1} I_2).

Protease activity

F. verticillioides exoantigen protease activity was analyzed according to Heussen and Dowdle (1980) with some modifications.

Briefly, *F. verticillioides* 97K exoantigen was mixed with sample buffer (7.5% v/v SDS; 3% v/v sucrose (Sigma, Saint Louis, USA); 12 µg mL^{-1} bromophenol blue) and was submitted to the SDS-PAGE in a 10% resolving gel containing 0.1% v/v gelatin or 0.1% v/v BSA. The electrophoresis was carried out at 100 V at 4 °C until the dye reached the end of the gel. The gel was then washed with 2.5% Triton X-100 at 25 °C for 1 h and was incubated with 0.1 mol L^{-1} glycine pH 8.3 at 37 °C for 4 h. After this period, the gel was stained with Coomassie Brilliant Blue. Trypsin was used as positive control.

Results and Discussion

The exoantigen of *F. verticillioides* 97K showed a concentration of 1.5 mg mL^{-1}. After sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining, it was possible to distinguish the p67 band (Figure 1) from the other bands. The bovine serum albumin (BSA) showed some extra bands, probably due to its degradation, but 67 kDa band remained the most abundant (Figure 1). Protein band densitometry estimated that p67 represents 30% of the total proteins in the *F. verticillioides* 97K exoantigen. According to previous studies carried out by our group, an indirect competitive ELISA using antibodies specific to a 67 kDa protein (p67) present in *F. verticillioides* exoantigen was able to detect contamination of poultry feed samples by this fungus (Omori et al., 2019). Therefore, in this study p67 was analyzed by Mass Spectrometry and identity with two possible proteins were identified by database search, the protein related to the aminopeptidase Y precursor of *F. fujikuroi* and the putative glucoamylase GMY2 of *F. verticillioides.*

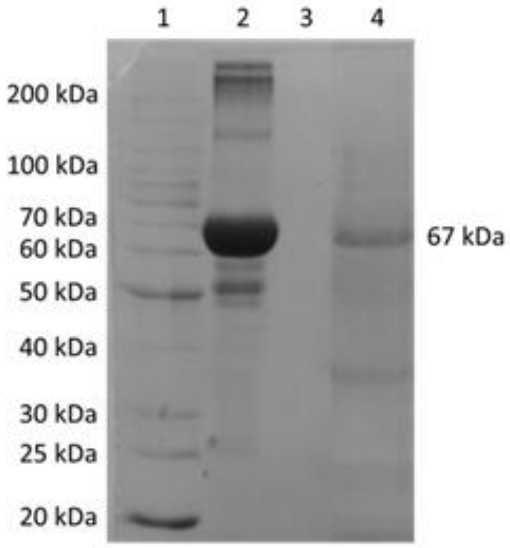

Figure 1. Electrophoretic pattern of the *F. verticillioides* 97K exoantigen and bovine serum albumin (BSA) in polyacrylamide gel stained by Coomassie Brilliant Blue.
1. Molecular weight standard; 2. BSA; 3. Empty; 4. *F. verticillioides* 97K exoantigen.

Approximately 1 mmol of p67 were detached from the gel and submitted to in-gel trypsin digestion. The analysis of the in-gel trypsin digested p67 by UPLC-MS/MS and the subsequent research in NCBI database using Mascot resulted in two possible proteins, the protein related to the aminopeptidase Y precursor of *F. fujikuroi* (Accession number: CCT71037) with score of 1502 and the putative glucoamylase GMY2 of *F. verticillioides* (Accession number: ABY89281) with score of 989. Nineteen peptides generated by the digestion of p67 matched with the peptides present in the protein related to the aminopeptidase Y precursor covering 50% of its amino acid sequence. On the other hand, the putative glucoamylase GMY2 showed 34% of its amino acid sequence covered by sixteen peptides generated by the digestion of p67.

In the negative control, which consisted of gel digested with trypsin without any protein, only *Bos taurus* cationic trypsin (Accession number: P00769) was identified with score of 2167. Five peptides generated by negative control digestion covered 27% of the total amino acid sequence of identified protein. And as expected, the in-gel trypsin digested BSA was identified as serum albumin of *Bos taurus* (Accession number: P02769) with score of 3669. Nineteen peptides generated by BSA digestion in gel covered 29% of the amino acid sequence of the identified protein. These results indicate that there was no contamination during the sample processing and that the data obtained are reliable.

The protein related to the aminopeptidase Y precursor of *F. fujikuroi* is composed of 491 amino acids and contains a region similar to the protease-associated domain and polypeptide binding site suggesting that this protein shows protease activity (Accession number: CCT71037). This protein has an identity of 99% with the aminopeptidase of *F. verticillioides* (Accession number: XP_018752314) which is also composed of 491 amino acids suggesting that p67 could be the aminopeptidase of *F. verticillioides.*

The putative glucoamylase GMY2 of *F. verticillioides* contains 583 amino acids and includes a starch-binding domain and two starch-binding sites which indicate that this protein shows amylase activity (Accession number: ABY89281).

For a reliable identification of p67, amylase and protease activity of this protein was analyzed using polyacrylamide gels copolymerized with starch, gelatin and BSA. p67 showed amylase activity and did not show protease activity confirming that this protein is the putative glucoamylase GMY2 of *F. verticillioides* (Figure 2). Protease activities were observed in the 44 kDa and 83 kDa proteins of *F. verticillioides* exoantigen in polyacrylamide gel copolymerized with gelatin and BSA, respectively (Figures 3 and 4).

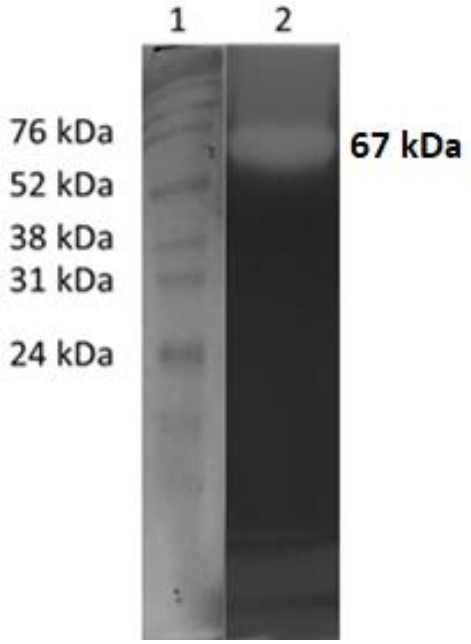

Figure 2. Amylase activity of the proteins from the *F. verticillioides* 97K exoantigen in polyacrylamide gel copolymerized with starch.
1. Molecular weight standard; 2. *F. verticillioides* 97K exoantigen.

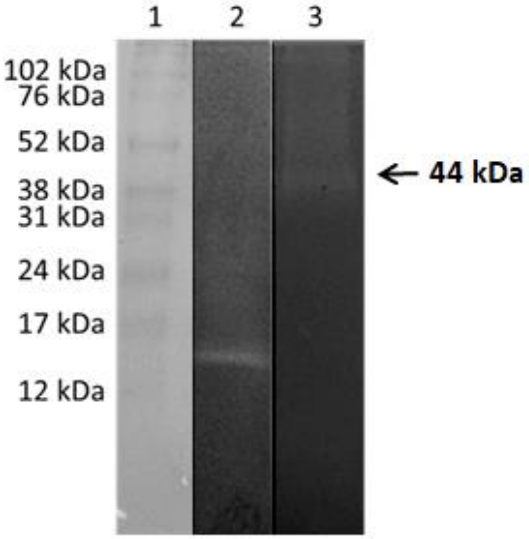

Figure 3. Protease activity of the proteins from the *F. verticillioides* 97K exoantigen in polyacrylamide gel copolymerized with gelatin.
1. Molecular weight standard; 2. Trypsin; 3. *F. verticillioides* 97K exoantigen.

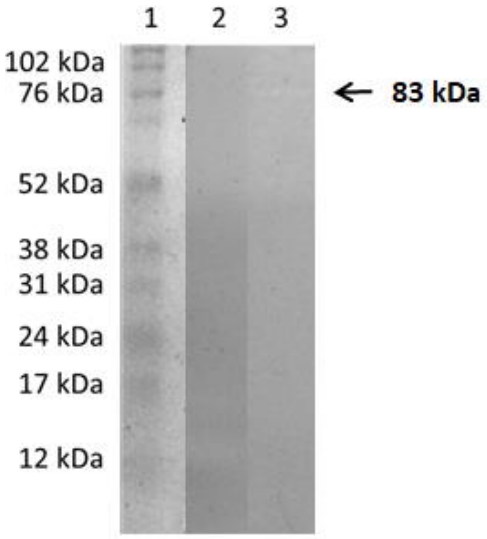

Figure 4. Protease activity of the proteins from the *F. verticillioides* 97K exoantigen in polyacrylamide gel copolymerized with bovine serum albumin (BSA).
1. Molecular weight standard; 2. Trypsin; 3. *F. verticillioides* 97K exoantigen.

The in-gel analysis of p67 showed only amylase activity, confirming that this protein is the putative glucoamylase GMY2 of *F. verticillioides.* Glucoamylase and amylase represent the two major classes of starch-degrading enzymes identified in fungi. Glucoamylase hydrolyses single glucose units from the non-reducing ends of amylose and amylopectin in a stepwise manner, while the α-amylase randomly hydrolyses the 1,4-α-D-glycosidic links between adjacent glucose residues in linear amylose chains (Krause et al., 1991). Bluhm and Woloshuk (2005) observed that kernels lacking starch due to physiological immaturity did not accumulate fumonisin B1 and demonstrated the importance of starch degradation by amylase activity in fumonisin B_1 synthesis working with a mutant strain of *F. verticillioides* with a disrupted α-amylase gene. They showed that the amylopectin, a component of starch, induces fumonisin B_1 production by *F. verticillioides.* Therefore, the high correlation between the fumonisin and p67 levels (r = 0.76) (now known to be the putative glucoamylase GMY2 of *F. verticillioides*) observed in the previous study (Omori et al., 2019) could be explained by the influence of amylase activity on the fumonisin synthesis pathway.

The complete coding sequence of the putative glucoamylase GMY2 gene of *F. verticillioides* (Accession number: EU247509.1) is already available which facilitates the production of the recombinant protein.

Conclusion

p67 of the *F. verticillioides* exoantigen was identified as putative glucoamylase GMY2 by UPLC-MS/MS and enzymatic study in polyacrylamide gel. This identification

will allow the large-scale production of the recombinant protein and the development of an immunological method specific to toxigenic *F. verticillioides* strains, thus contributing to minimize fumonisin contamination and improve food quality and safety.

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