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ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · JANUARY 2001

Impact Factor: 2.91 · DOI: 10.1021/jf0005770 · Source: PubMed

Interaction of *Fusarium* Mycotoxins, Fusaproliferin and Fumonisin B_1, with DNA Studied by Electrospray Ionization Mass Spectrometry

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Electrospray ionization mass spectrometry (ESI-MS) in negative ion mode was used to monitor the possible noncovalent adduct formations between DNA analogue oligonucleotides and two *Fusarium* mycotoxins, fumonisin B_1 and fusaproliferin. Using mild experimental ESI conditions specific noncovalent interactions were detected between both single- and double-stranded model oligonucleotides and fusaproliferin with 1:1 stoichiometry. Similar association complexes were observed for the deacetyl derivative of fusaproliferin. There were no peaks due to adduct formation present in the mass spectra of fumonisin B_1, incubated with oligonucleotides in a wide concentration range, suggesting no specific interaction for this molecule. In a competitive complexation reaction, another mycotoxin, the beauvericin, forms more stable association complex with DNA than fusaproliferin. These findings can be of use in the understanding of molecular mechanisms of action during apoptosis and can be correlated with the teratogenic effect of fusaproliferin.

Keywords: *Mycotoxins; fumonisin B_1; fusaproliferin; beauvericin; ESI-MS; oligonucleotides; noncovalent interaction*

INTRODUCTION

Fumonisin B_1 is the most important and well-studied member of fumonisins (FBs) family isolated from *Fusarium moniliforme* (Gelderblom et al., 1988). FBs are several structurally related compounds derivatives of diesters of propane-1,2,3-tricarboxylic acid and 2-amino-12,16-dimethyl polyhydroxyeicosane (Nelson et al., 1993). The structure of fumonisin B_1 (Figure 1) resembles sphingoid bases and thus it is a potent inhibitor of the spinganine-*N*-acetyltransferase, a key enzyme of sphingolipid biosynthesis (Wang et al., 1991; Prelusky et al., 1996). Fumonisin B_1 has been detected in maize and maize-based products in many areas (Marasas, 1995; Visconti et al., 1995; Visconti, 1996) and associated with regions with high incidence of human esophageal cancer (Voss et al., 1990). Fumonisin B_1 causes a number of toxicoses in animals, including equine leukoencephalomalacia (Gelderblom et al., 1988) and porcine pulmonary edema (Harrison et al., 1990), hepatotoxicity (Gelderblom et al., 1996), nephrotoxicity, and genotoxity (Knasmuller et al., 1997). Cytotoxicity of fumonisin B_1 was explained by lipid peroxidation and by the inhibition of cellular synthesis of macromolecules, proteins, and DNA (Abado-Becognee et al., 1998; Sahu et al., 1998). Fumonisin B_1-mediated apoptosis was observed in a variety cell types and tissues (Tolleson et al., 1999; Schmelz et al., 1998; Zhang et al., 1999; Atroshi et al., 1999). Apoptotic activity of fumonisin B_1 was explained by the disruption of sphingolipid biosynthesis (Tolleson et al., 1999). Fumonisin B_1-induced apoptosis in CV-1 cells was demonstrated to be induced by the tumor necrosis factor pathway in which caspase was proved to have an important role (Ciacci-Zanella et al., 1999).

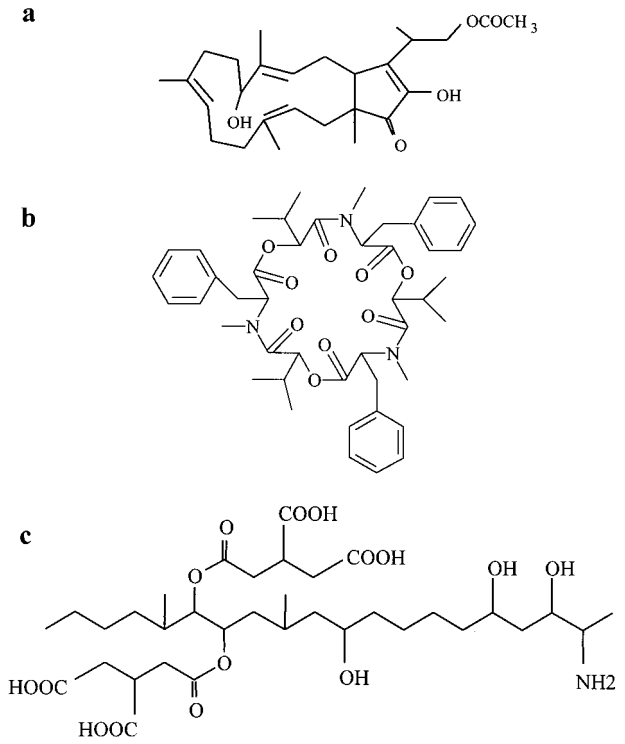

Figure 1. Chemical structures of mycotoxins studied: (a) fusaproliferin, (b) beauvericin, and (c) fumonosin B_1.

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10.1021/jf0005770 CCC: $19.00
Published on Web 11/14/2000

In addition, fumonisin B_1 was found nonmutagenic in the Salmonella assay and is not teratogenic in rat pregnancy assay (Collins et al., 1998).

Electrospray ionization mass spectrometry (ESI-MS) is a well-established method to obtain molecular mass and structural information on a wide variety compounds starting from medium sized organic molecules to higher mass proteins and oligonucleotides. The primary utility of electrospray lies in its ability to produce singly and/or multiply charged gaseous ions directly from an aqueous or aqueous/organic solvent system using mild experimental conditions in order to detect nonvolatile molecules without considerable fragmentation (Loo and Loo, 1997). In the past decade the technique was found valuable in the studies of higher order protein structures and weakly bound complexes as well. Experimental evidence suggests that the ESI-MS observations of biocomplexes reflect to some extent the nature of the interaction present in the condensed phase (Siuzdak et al., 1996; Loo, 1997). Association complexes, where the presence of specific interaction was already known in solution, have been observed in the gas phase among oligonucleotides (Ganem et al., 1993; Goodlett et al., 1993), peptides (Li et al., 1993; Smith and Zhang, 1994; Pramanik et al., 1998), and proteins (Light-Wahl et al., 1994; Huang et al., 1993; Loo et al., 1998). The mass accuracy of the molecular weights determined for the intact complexes by ESI-MS is far more superior to the traditional methods of gel permeation chromatography or sodium dodecyl sulfate polyacrilamid gel electrophoresis. Stoichiometry of the complexes can be obtained directly from the mass spectrum (Cheng et al., 1996), and ESI-MS offers an advantage to conventional methods in terms of sensitivity and speed too. Specific heteromolecular interactions of oligonucleotides have been described with various small ligands, like distamycins (Gale et al.; 1994; Triolo et al., 1997), actinomycin D (Hsieh et al., 1994), and beauvericin (Pocsfalvi et al., 1997). Applying ESI-MS for the detection of such weak complexes is a straightforward and sensitive way for the screening of antitumor drugs binding to DNA (Gale and Smith, 1995; Gabelica et al., 1999). The method has also the potential to reveal information on action mechanism of mycotoxins in biological systems (Pocsfalvi et al., 1997).

Two *Fusarium* mycotoxins, fumonisin B_1 and fusaproliferin, were chosen for this study (Figure 1). Fusaproliferin was first isolated from *Fusarium proliferatum* (Randazzo et al., 1993) and then *F. subglutinans* cultures (Logrieco et al., 1996). It was found toxic in various cell lines (Randazzo et al., 1993; Kostecki et al., 1999). Severe teratogenic effects, such as cephalic dichotomy, macrocephaly, limb asymmetry, hemorrhages, and incomplete closure of the umbilicus were attributed to fusaproliferin in a chicken embryotoxicity bioassay (Ritieni et al., 1997a). Recently, fusaproliferin was showed to increase the sister chromatid exchanges and chromosome aberrations in mitotic chromosomes of the goat (Di Berardino et al., 1999).

Recently, the formation of noncovalent complexes between oligonucleotides and another mycotoxin with ionophore-induced apoptotic properties, the beauvericin (Figure 1), was observed using ESI-MS in negative ion mode (Pocsfalvi et al., 1997). Segmentation of the DNA, which occurs during apoptosis, at least partly has been correlated to the noncovalent interaction of beauvericin with DNA. The aim of this study was to apply ESI-MS to the mixtures of oligonucleotide and mycotoxins to monitor possible noncovalent interactions and to establish the nature of the interaction.

MATERIALS AND METHODS

Materials. A 6-mer self-complementary oligonucleotide ($^{5'}$-GTTAAC$^{3'}$, MW 1786.3) was synthesized by Ceinge Biotecnologie Avanzate (Naples, Italy). Non self-complementary 14-mer oligonucleotide ($^{5'}$ATCGTCACGGCGAT$^{3'}$, MW 4250.8) and 12-mer polyoligonucleotides (poly-T, poly-G, poly-A, and poly-C) were purchased from Genosys Biotechnologies (Cambridge, England). Oligonucleotides were purified by reverse-phase HPLC. Fusaproliferin sesterterpene ($C_{27}H_{40}O_5$, MW 444) was isolated and purified according to Ritieni et al., 1997b. Fumonisin B_1 (MW 721) from *Fusarium moniliform* was purchased from Sigma Chemical Co., St. Louis, MO, and was used without further purification. Beauvericin (cyclo[D-α-hydroxy-isovaleryl-L-*N*-methylphenylalanyl]$_3$, MW 783.4) was isolated from *Fusarium subglutinans* (Pocsfalvi et al., 1997).

Incubation with Oligonucleotides. Oligonucleotides were dissolved in sterile twice-distilled water and their concentrations was determined by UV spectrophotometry. Annealing of 6-mer self-complementary oligonucleotide was performed in ammonium acetate at pH 8, heating the solution to 95 °C and then slowly (over 3 h) cooling it down to room temperature. Fusaproliferin and fumonisin B_1 were dissolved in methanol obtaining a stock solution of 2.5 nmol/μL. The solutions of oligonucleotide and mycotoxin were mixed together in a polypropylene Eppendorf tube at ambient temperature so as to keep the mixtures in 75% (v/v) methanol. The molar ratio was kept 1:1 (when otherwise is not stated) while different concentrations were made up for individual experiments.

Mass Spectrometry. A platform LC single-quadrupole mass spectrometer (Micromass, Altrincham, UK) was used in the negative ion mode for all experiments. Aliquots (10 μL) were introduced through a Rheodyne external loop injector into the ion source at a flow rate set to 10 μL/min using a Phoenix 20 CU HPLC pump. Different buffer solutions were tried in order to obtain maximum signals for the adduct ions. Methanol/water 1:1 with 0.1% (v/v) NH_3 was found to be the best for this purpose. Spraying was achieved by using nitrogen as the nebulizing gas with a probe voltage of −2.8 kV. The cone voltage was set to −30 V and the source temperature to 40 °C, since with these parameters, optimum sensitivity was obtained for the peaks corresponding to the complex and no fragmentation was observed. Calibration of the mass scale was performed by using the multiply charged ions of horse heart myoglobin, from a separate sample introduction in the positive-ion mode. Full-scan mass spectra were acquired in continuous data-acquisition mode. The spectra shown here are an average of 5 scans from *m*/*z* 300 to 1300 at a scan rate 10 s/scan.

RESULTS

ESI mass spectrum of fusaproliferin in negative ion mode (Figure 2a) results in a base peak due to the singly charged molecular ion, $(F-H)^-$ at *m*/*z* 443. Deacetylation of fusaproliferin leads to the deacetyl derivative which appears in the spectrum at *m*/*z* 401, $(DaF-H)^-$. The intensity of this peak varied significantly depending on sample purities but was present in the spectrum even after rigorous purification steps. The relative intensity of $(F-H)^-/(DaF-H)^-$ peaks does not depend on the ion source parameters, suggesting that deacetylated fusaproliferin is present as impurities and not as a product ion formed as a result of fragmentation within the source or the skimmer region of the mass spectrometer. This observation was further confirmed by high-energy collision induced dissociation experiments performed on fusaproliferin $(F-H)^-$ showing no significant deacetylation upon fragmentation (data not shown). The ESI mass spectrum (Figure 2b) of the pure, 6-mer, self-

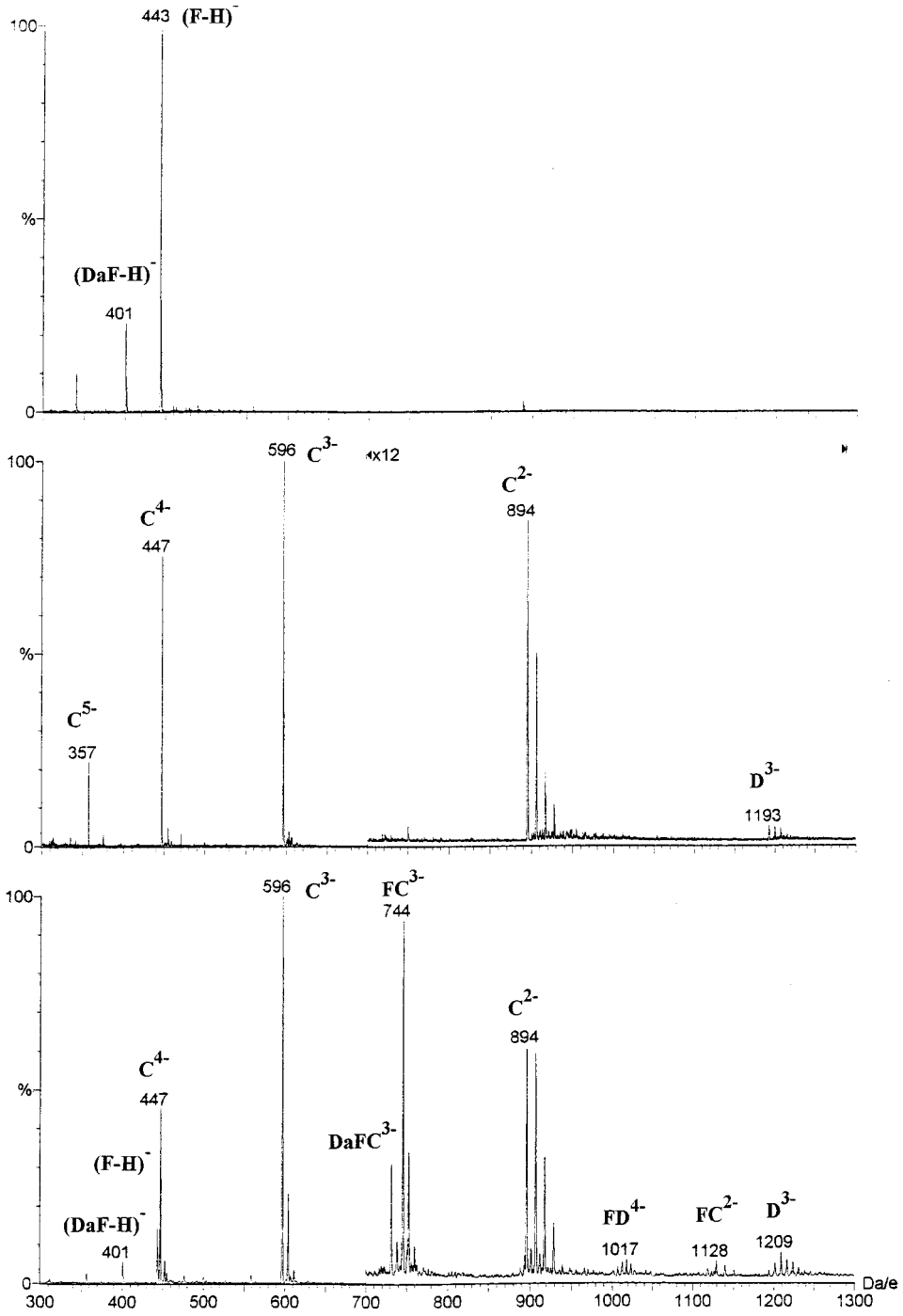

Figure 2. Electrospray negative ion mass spectrum of (a) fusaproliferin, (b) oligonucleotide 6-mer ($^{5'}$GTTAAC$^{3'}$), and (c) incubated solution of fusaproliferin and 6-mer oligonucleotide in 1:1 molar ratio at 62 pmol/μL concentration. Peaks corresponding to fusaproliferin and to oligonucleotide are assigned by F and C, respectively.

complementary annealed oligonucleotide ($^{5'}$GTTAAC$^{3'}$, MW 1786.3) yields a series of multiply charged peaks of the single-strand chain (C^{n-}, where n is the number of negative charges). Peaks in the higher mass range (lower charge state), like m/z 894 (C^{2-}) are accompanied by adduct peaks containing one or more of sodium ion ($C(Na)_x(n + x)^-$), which is a general characteristics of oligonucleotide spectrum even after rigorous desalification steps. The peak at m/z 1193 (D^{3-}) and its sodium adduct peaks nearby represent the duplex formation between the two complementary chains of the oligonucleotide (note that the duplex peaks at even charges, like D^{4-} and D^{6-}, are in coincidence with the monomer peaks $C^{2-}$and C^{3-}). The intensity of the duplex peak is relatively low, which suggests the major part of the duplex form present in the solution phase dissociates during the ionization and detection. Figure 2c shows the ESI mass spectrum of the same oligonucleotide incubated with fusaproliferin in a 1:1 molar ratio at a concentration of 62 pmol/μL. Besides the identified peaks of the components ($(F-H)^-$, $(DaF-H)^-$, C^{n-}, D^{3-}), new peaks representing the adduct formations between oligonucleotide and fusaproliferin can also be observed. Thus, abundant peaks at m/z 744 and 1128 are due to the single-stranded oligonucleotide with one molecule of fusaproliferin attached (FC^{3-} and FC^{2-} sodiated). Similarly, the deacetylated fusaproliferin also leads to a adduct peak at m/z 730 ($DaFC^{3-}$). Adduct formation

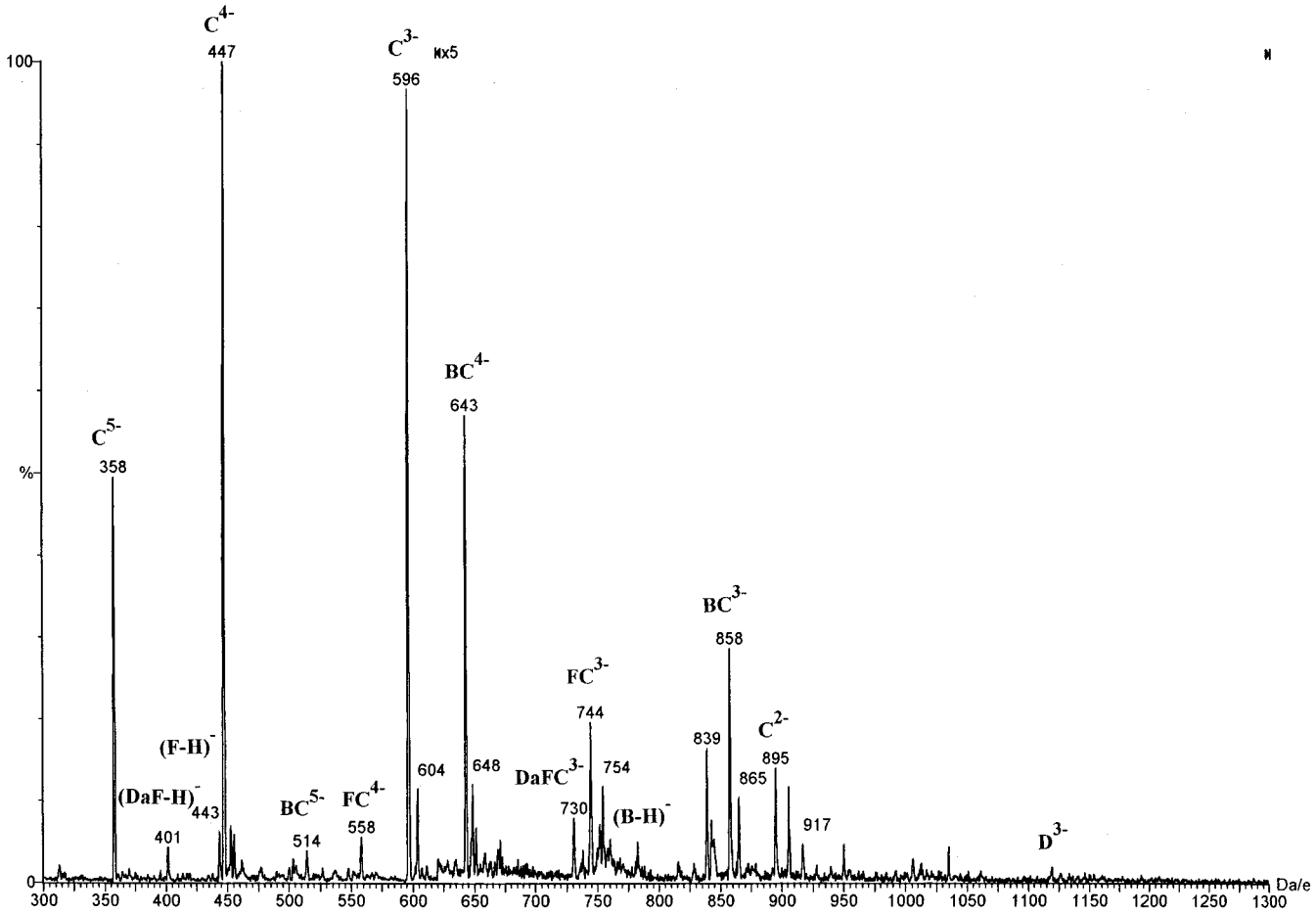

Figure 3. ESI negative ion mass spectrum of the incubated solution of fusaproliferin and beauvericin incubated with $^{5'}$GTTAAC$^{3'}$ oligonucleotide in 1:1:1 molar ratio at 125 pmol/μL concentration. Peaks corresponding to fusaproliferin, beauvericin, and to oligonucleotide are assigned F, B, and C, respectively.

between fusaproliferin and the duplex form of the oligonucleotide can be deduced from the presence of the peak at *m/z* 1006.0 and its nearby sodiated forms (FD^{4-}). The relative intensity of this peak is similar to that of D^{3-}, suggesting specific interactions between oligonucleotides and this mycotoxin. With use of higher cone voltage, duplex and adduct peaks disappear, showing a facile fragmentation of weakly bounded molecules. Various incubations followed by ESI-MS measurements were performed with different concentrations but maintaining the 1:1 molar ratio at 5, 15, 62, 125, 250, and 500 pmol/μL concentrations. Adduct formation was detected at concentrations as low as 15 pmol/μL. The ratio of relative abundance of adduct and oligonucleotide peaks only slightly increased by increasing the concentrations of monomers, in contrast to what was found previously in the beauvericin−oligonucleotide associations (Pocsfalvi et al., 1997). Stoichiometry of the associations was preferentially 1:1 at low incubation concentrations. Incubations performed at high concentrations (250 and 500 pmol/μL with 1:1 molar ratio) and incubation performed with high molar excess of fusaproliferin (10:1 molar ratio) adducts with 2:1 stoichiometry (two fusaproliferin molecules attached to one molecule of oligonucleotides) were also observed.

Fusaproliferin was also incubated with a 14-mer non-self-complementary oligonucleotide ($^{5'}$ATCGTCACGGCGAT$^{3'}$) and four 12-mer polyoligonucleotides (poly-C, poly-G, poly-A, and poly-T). Adduct ions in the ESI spectrum of these mixtures were also observed (data not shown here). The selectivity of binding of fusaproliferin to different 12-mer polyoligonucleotides was found to be similar, indicating that fusaproliferin does not have a strongly preferred base sequence or base site in the DNA.

Competitive complexation reaction between two mycotoxins was performed incubating the 6-mer oligonucleotide ($^{5'}$GTTAAC$^{3'}$) with fusaproliferin and beauvericin (MW 783.4) in 1:1:1 molar ratio at 125 pmol/μL concentration. The resulting spectrum (Figure 3) shows adduct peaks with both mycotoxins. Complex peaks of beauvericin (BC^{5-}, BC^{4-}, and BC^{3-}) are more abundant than the corresponding adduct peaks of fusaproliferin (FC^{4-} and FC^{3-}).

Electrospray mass spectra of fumonisin and its synthetic derivatives have been reported (Caldas et al., 1995; Josephs, 1996). ESI in negative ion full scan mode results in sensitive and selective detection of low picomolar levels of fumonisin B_1 because of abundant $(M-H)^-$ and little fragmentation. Two abundant peaks at *m/z* 360 and 720 corresponding to the doubly and singly charged deprotonated molecular ions of FB_1 are $(FB-2H)^{2-}$ and $(FB-H)^-$ (Figure 4a). Fumonisin B_1 was incubated with both the 6-mer self-complementary and the 14-mer single-stranded oligonucleotides under various experimental conditions, such as different concentrations in the range of 15−500 pmol/μL at a fixed molar ratio of 1:1, different molar ratios at 250 pmol/μL concentration (fumonisin B_1:oligonucleotide = 10:1, 5:1, 2:1, and 1:1), and different incubation times (from a few minutes to one month). The incubated solutions were analyzed by ESI-MS using the same experimental

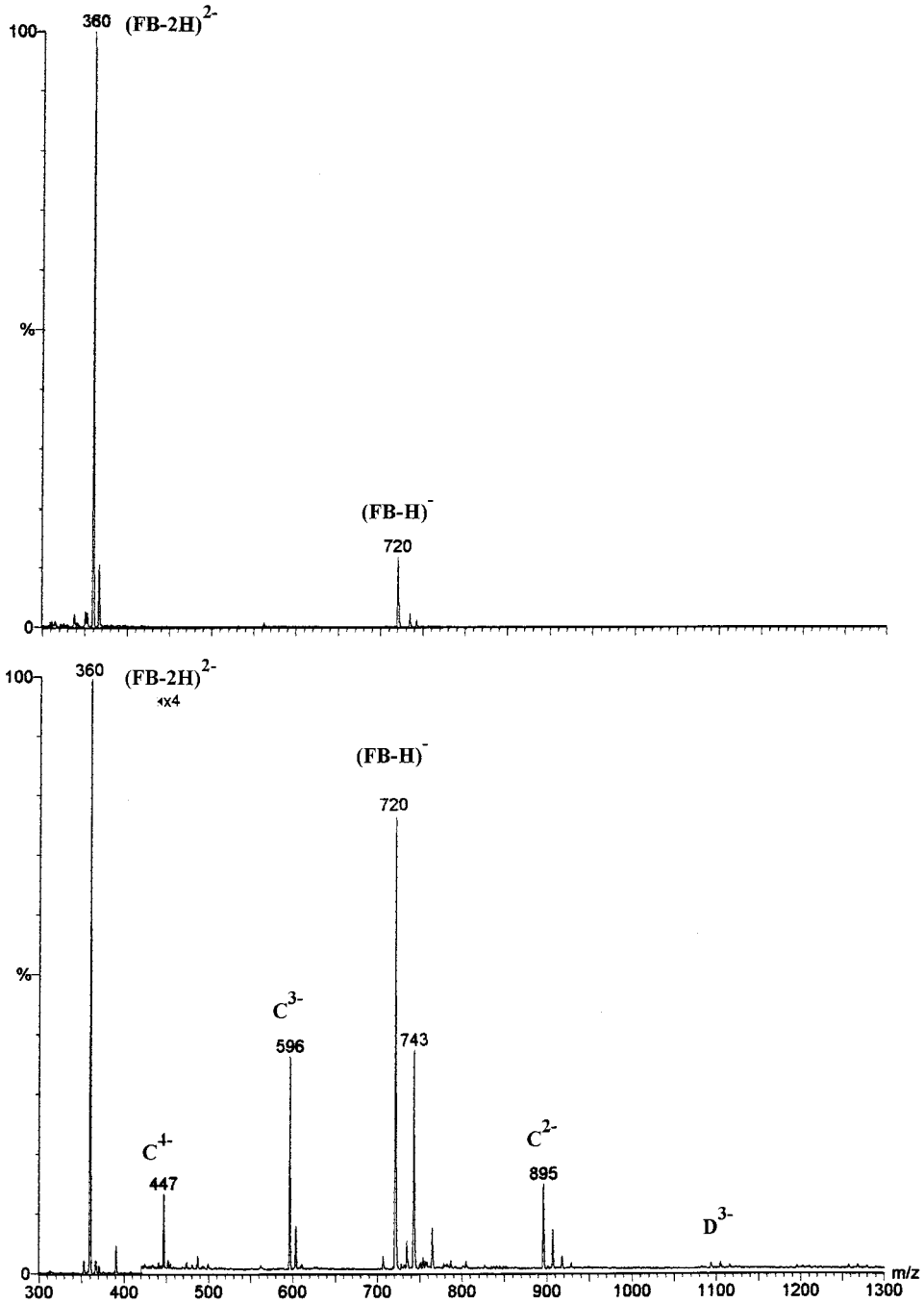

Figure 4. ESI negative ion mass spectrum of (a) fumonisin B_1 and (b) incubated solution of fumonisin B_1 and oligonucleotide $^{5'}$GTTAAC$^{3'}$ in 1:1 molar ratio at 250 pmol/μL concentration. Peaks corresponding to fumonosin B_1 and to oligonucleotide are assigned FB and C, respectively.

parameters as were used for fusaproliferin and beauvericin. No adduct formations could be observed with this mycotoxin (Figure 4b). ESI-MS experiments performed on the 1:1:1 mixture containing fusaproliferin, FB_1, and oligonucleotides (spectra not shown) yield adduct formation only between oligonucleotide and fusaproliferin.

DISCUSSION

Studying *Fusarium* mycotoxins and their possible noncovalent interaction with DNA it was found that fusaproliferin similarly to beauvericin forms weakly bound complexes with various oligonucleotides. The formation of such adducts is specific for both single- and double-stranded oligonucleotides but is not specific for a determined sequence or base type. These suggest an electrostatic type of interaction between the negatively charged sugar−phosphate backbone and the fusaproliferin molecule. In a competition complexation reaction where a model oligonucleotide was incubated with a 1:1 mixture of fusaproliferin and beauvericin, the later showed higher affinity. Fumonisin B_1 was also investigated in the same manner and no adduct formation could be detected, suggesting the lack of noncovalent interaction with DNA for this mycotoxin.

The results obtained by mass spectrometry experiments can be correlated with the different biological properties of the mycotoxins (apoptotic, cytotoxic, and teratogenic activities) to gain a better insight of their mechanisms of action at molecular level. Beauvericin,

fusaproliferin, and fumonisin B_1 are different in their chemical structures and consequently in their biological activities. Potassium ionophore beauvericin induces a type of cell-death very similar to apoptosis due to tumor necrosis factor (TNFα) accompanied by the segmentation of DNA. The apoptotic activity of beauvericin was correlated with the increase in the cytoplasmic calcium concentration released from intracellular stores (Ojcius et al., 1991) and with its ability to form noncovalent association complexes with DNA (Pocsfalvi et al., 1997). Fumonisin B_1 is known to open different channels for the execution of the apoptotic process leading to sphinganine accumulation and ceramide depletion (Tolleson et al., 1999). A recent study performed in monkey kidney cells demonstrates that FB_1 transcriptionally activates the *p21* promoter through two Sp1 related proteins (Zhang et al., 1999). The exact mechanism of activation is not clear but it was supposed that the activity is possibly mediated by a posttranslational modification of Sp1 factors. Thus in the case of FB_1 the initial interaction more likely occurs between a protein and mycotoxin and not between DNA and mycotoxin. The apolar sesterterpene structure of fusaproliferin may permit a direct cross of the lypidic cell membranes. Reaching the cell nucleus, it is tempting to speculate that fusaproliferin can interact noncovalently with DNA and modify gene regulation during the development of an embryo, which can be the cause of the reported teratogenic effects due to a long exposure to this mycotoxin in biological model systems.

Evidence is building worldwide implicating these mycotoxins in animal and human diseases and there is a parallel effort for their identification, sensitive detection, and quantification. Carefully designed in vitro ESI-MS experiments can yield observation of weak biomolecular interactions of mycotoxins with biomacromolecules, like proteins and DNA, providing additional information for the evaluation of health risks related to the consumption of mycotoxin contaminated food. A further development in the evaluation of the competitive complexation reaction monitored by ESI-MS can lead an in vitro assay for risk assessment of synergic and/or addition effects due to two or more toxins together.

ABBREVIATIONS USED

ESI-MS, electrospray ionization mass spectrometry; HPLC, high-performance liquid chromatography.

SAFETY

Fumonisin B_1, fusaproliferin, and beauvericin are toxic compounds and should be handled with extreme caution.

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Received for review May 12, 2000. Revised manuscript received August 3, 2000. Accepted August 4, 2000. This work was supported by EU Grant ERB FMGE CT95-0061, Fondo Europeo di Sviluppo Regionale, FERS No. 94.05.09.013, ARINCO No. 94.IT.16.028, and Hungarian-Italian intergovernmental S&T cooperation program, 1998−2000.

JF0005770