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Fumonisin elimination and prospects for detoxification by enzymatic transformation

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Abstract

A technology to efficiently reduce the concentration of carcinogenic and toxic fumonisins in food and feed would be desirable. This class of mycotoxins is produced by the maize pathogen *Fusarium verticillioides* and other fungi. Fumonisins are frequently found in maize from the warm growing regions of the world, sometimes in considerable concentrations. Their molecular similarity with sphingolipids enables their binding to mammalian ceramide synthase, and the resulting interference with sphingolipid metabolism. Recently, we reported on a cluster of genes of *Sphingopyxis* sp. MTA144 which enables this alphaproteobacterium to degrade fumonisins. These and the previously known fumonisin catabolism genes and enzymes from the black yeast *Exophiala spinifera* and from bacterium ATCC 55552 allow the consideration of prospects for enzymatic detoxification of fumonisins in food and feed. All the known fumonisin catabolism pathways start by hydrolytic release of the two tricarballylic acid side chains, followed by removal of the 2-amino group from the core chain by different enzymatic mechanisms. The potential for application of feed enzymes for fumonisin detoxification in the gastrointestinal tract of animals is discussed, and possible applications in processing of maize for feed or food are also considered. To be able to evaluate the requirement for, and potential of, a new, enzyme-based fumonisin detoxification technology, an overview of the state of the art of fumonisin elimination and the known chemical reactions of fumonisins in processing or decontamination is also given. There is a special focus on the toxicity of hydrolysed fumonisins, because they can be generated from fumonisins both by an established, traditional method of maize processing, nixtamalisation, and by enzymatic biotransformation. As a complement to other approaches, enzymatic degradation of fumonisins to ameliorate the health risk of contaminated maize for animals, and possibly also for humans, seems feasible.

Keywords: fumonisin, carboxylesterase, aminotransferase, maize, enzyme, toxicity

1. Introduction

Contamination of maize with fumonisins is only one aspect of an overwhelming challenge that agriculture is facing in the 21st century. The growing world population will need more food, but increasing the amount of land available for cultivation of crops is difficult because of the need to preserve natural ecosystems, competition with other uses of land, or because it is costly (Godfray *et al.*, 2010). The use of crop plants for biofuels and the demand for meat, dairy products and fish, which correlates with increasing wealth and which requires the use of agricultural commodities for animal feed, will further elevate the need for more primary agricultural produce. In addition, climate change will set new challenges for the food supply chain (Schmidhuber and Tubiello, 2007). Many crop diseases have been successfully mitigated by plant breeding and by adaptation of agricultural practices in the past, but with so many other agricultural challenges to focus on, the problem of susceptibility of maize to *Fusarium verticillioides* and *Fusarium proliferatum* infection, and the resulting contamination with fumonisins, may not be possible to solve in the near future. As long as the formation of fumonisins in crop plants cannot be prevented, strategies for elimination and detoxification should be considered to increase the safety and nutritional value of contaminated agricultural commodities. The guidance values or maximum levels, which were established by the US Food and Drug Administration, the European Union, and many other countries, for fumonisins in certain foods and feeds were

based not only on toxicological data, but also on the levels that were judged to be achievable with the use of good agricultural and good manufacturing practices. Therefore, fumonisin concentrations below guideline or legal limits, at least in feed, cannot necessarily be considered harmless. Furthermore, a proportion of the world's maize is never traded and never analysed for fumonisin contamination, because it is eaten by subsistence farmers, or because, also in industrial countries, it is used for animal production on the farm where it was grown. For these reasons, the fumonisin problem cannot be solved by regulating authorities alone, so technological solutions for fumonisin elimination must also be considered.

Technological possibilities for fumonisin elimination include physical separation of the most highly contaminated fractions, binding to certain adsorption agents in the gastrointestinal tract to reduce bioavailability, and conversion of fumonisins, chemically or enzymatically catalysed, to less toxic metabolites. Even though the fumonisins are quite stable molecules, several fumonisin-consuming reactions have been described, which can take place as decontamination reactions or during certain food processing steps such as nixtamalisation, baking, frying or extrusion. Microbial genes and enzymes for catabolism of fumonisins have previously been described (Blackwell *et al.*, 1999), and their potential for fumonisin detoxification in transgenic plants has been discussed (Duvick, 2001). Recently, we reported bacterial enzymes, including ones that catalyse a new reaction in fumonisin catabolism (Heinl *et al.*, 2010, 2011). An application of such enzymes, applied in the course of food or feed processing, or as animal feed additive for reaction in the gastrointestinal tract, seems feasible. The prospects for a future technological application, including challenges and safety considerations, are discussed in the present article. However, a new technology can only be evaluated in comparison with the state of the art, and enzymes would need to complement existing or alternative methods. For these reasons, an overview of the current knowledge about non-enzymatic fumonisin elimination is also given.

2. Occurrence and toxicity of fumonisins

Fumonisin-producing *Fusarium* species, especially *F. verticillioides* and *F. proliferatum*, are ubiquitous in the climatically warm maize-growing regions of the earth. The fumonisin concentrations in maize and maize products from many countries were analysed and reported (Binder *et al.*, 2007; Castelo *et al.*, 1998b; Monbaliu *et al.*, 2010; Shephard *et al.*, 1996). Fumonisins were first isolated from *F. verticillioides* (Bezuidenhout *et al.*, 1988; Gelderblom *et al.*, 1988), and in the meantime several structurally related variations were found to be produced by *Fusarium* species (Rheeder *et al.*, 2002), *Aspergillus niger* (Frisvad *et al.*, 2007), *Alternaria alternata* (Chen *et al.*, 1992) and *Tolypocladium* species (Mogensen *et al.*, 2010). The molecular mechanism of toxicity of fumonisins, the inhibition of the enzyme ceramide synthase (Wang *et al.*, 1991), has been well studied and reviewed before (Merrill *et al.*, 2001; Riley *et al.*, 2001). The resulting wide range of toxic effects and mycotoxicoses induced by fumonisins, including equine leukoencephalomalacia (Kellerman *et al.*, 1990; Marasas *et al.*, 1988), porcine pulmonary oedema (Harrison *et al.*, 1990; Haschek *et al.*, 2001), carcinogenicity (Gelderblom *et al.*, 1991, 1992), induction of neural tube disorder (Gelineau-Van Waes *et al.*, 2009; Marasas *et al.*, 2004), immunomodulation and damage to the gastrointestinal tract (Bouhet and Oswald, 2007) have also been well covered in previous review papers (Diaz and Boermans, 1994; Stockmann-Juvala and Savolainen, 2008; Voss *et al.*, 2007).

3. Fumonisin elimination

Physical separation

Manual sorting of maize may be the most effective post-contamination measure to reduce fumonisin intake in subsistence farming population, where fumonisin concentrations in the maize staple are often high (Van der Westhuizen *et al.*, 2010). Of the routine steps that can be included in industrial food or feed processing, physical separation is probably also the most efficient method of reducing fumonisin contamination. In intact kernels, fumonisin concentrations were reported to be much lower than in screenings (Murphy *et al.*, 1993; Sydenham *et al.*, 1994). Both dry and wet milling can be used to segregate fumonisins in the bran and germs fractions, which are used as animal feed, or the steeping water, respectively, to make cornmeal, flour, grit or starch containing little or no fumonisins (Bennett *et al.*, 1996; Katta *et al.*, 1997).

Adsorption

Adsorption of fumonisins to certain binding agents has been investigated for a possible application in animal nutrition. This approach has been very successful with aflatoxins, which show good binding to some selected, but not all, clay minerals that can be used as feed additives (Phillips, 1999). However, binding of fumonisins to bentonite, celite or activated carbon (Piva *et al.*, 2005; Solfrizzo *et al.*, 2001a,b) was insufficient for detoxification. Cholestyramine, which showed good binding (Avantaggiato *et al.*, 2005; Solfrizzo *et al.*, 2001b), has, to our knowledge, not been tested in farm animals or been implemented as a fumonisin-adsorbing feed additive. Binding of fumonisins to lactic acid bacteria was also investigated (Niderkorn *et al.*, 2006, 2007, 2009).

Chemical reactions in food or feed processing steps

A comprehensive compilation of the available experimental results on the stability of fumonisins in thermal food processing was written by Humpf and Voss (2004). The fumonisins are quite heat-stable molecules and decontamination efficiency strongly depends on temperature, pH, and processing time. In early work, no reduction of fumonisin B_1 (FB_1) concentration was observed when culture material of *F. verticillioides* was boiled in water for 30 min and dried at 60 °C for 24 h (Alberts *et al.*, 1990). However, fumonisin concentrations can be reduced by exposure to higher temperatures, as a number of subsequent reports have shown. The reaction rate constants of FB_1 at temperatures from 125 °C to 200 °C at pH 4.0, 7.0 and 10.0 were determined and the half-lives were reported to depend on pH and range from 375 min to 3 min (Jackson *et al.*, 1996). Dupuy *et al.* (1993) calculated the half-lives of FB_1 in dried corn that was exposed to temperatures in the range of 75 °C to 150 °C and reported half-lives from 8 h to 10 min in this temperature range. Scott and Lawrence (1994) confirmed by showing that heating of spiked corn meal to 220 °C for 25 min caused a complete loss of the fumonisins, but the same treatment of corn muffin mix, or heating of corn meal at lower temperature, caused only partial loss. These results show that baking or frying of corn-based foods can reduce the fumonisin content, if a sufficiently high temperature is applied. However, Jackson *et al.* (1997) reported that frying of spiked corn masa at 140 °C to 170 °C for up to 6 min failed to degrade FB_1, and frying temperatures over 180 °C and times over 8 min were required for reduction of fumonisin concentrations. Baking of corn muffins spiked with FB_1 at 175 °C or 200 °C resulted in only small reductions in fumonisin concentrations. Castelo *et al.* (1998c) also reported that baking a corn muffin mix did not, but roasting cornmeal samples at 218 °C did, cause loss of fumonisins.

Another high temperature processing method, which is used for the production of breakfast cereals or snack foods, is extrusion cooking. Steam and extrusion screws mounted in barrels are used to generate high temperature and pressure for a short time of exposure. There are several reports showing that extrusion can cause a partial loss of detectable fumonisins (De Girolamo *et al.*, 2001; Meister, 2001; Piñeiro *et al.*, 1999), depending on the moisture of the dough and the type of screw used (Castelo *et al.*, 1998a) as well as on barrel temperature and screw speed (Katta *et al.*, 1999). The addition of salt or sucrose was reported to enhance reduction of FB_1 content (Castells *et al.*, 2009).

The effect of pH on the stability of fumonisins is especially important, because the traditional, Latin American process of nixtamalisation for making maize tortillas involves cooking maize in alkaline lime water, steeping overnight, removing the liquor, and washing the maize before grinding it to masa. Ester bonds, which link the two tricarballylic acid (TCA) side chains to the core chain of FB_1, generally undergo hydrolysis under acidic or alkaline conditions, and FB_1 can be converted to hydrolysed FB_1 (HFB_1) by incubation in potassium hydroxide solution at elevated temperature (Gelderblom *et al.*, 1993). To a small extent, this hydrolysis reaction can also take place in the mammalian intestinal tract (Fodor *et al.*, 2008; Shephard *et al.*, 1994). The first evidence that hydrolysis of fumonisins can take place during nixtamalisation came from the finding that in samples of food that had been processed by alkaline cooking, fumonisins were absent or present only at low concentrations (Sydenham *et al.*, 1991). Direct evidence for hydrolysis of FB_1 was subsequently reported (De La Campa *et al.*, 2004; Dombrink-Kurtzman *et al.*, 2000; Hendrich *et al.*, 1993; Palencia *et al.*, 2003; Saunders *et al.*, 2001; Sydenham *et al.*, 1995; Voss *et al.*, 2001). However, the reported proportions of FB_1 to HFB_1, and the sums of the two, in the masa and the liquor vary over a considerable range.

Ethanol fermentation of maize mash can be considered a feed processing step, because the by-product of ethanol, distillers dried grains and solubles (DDGS), is used as animal feed. However, FB_1 is not degraded during the fermentation and accumulates in the distillers grains (Bothast *et al.*, 1992). Fumonisins that were spiked to wort before fermentation were found in beer in only slightly reduced concentrations (Scott *et al.*, 1995).

Chemical decontamination

Pioneering work on chemical decontamination of agricultural commodities containing mycotoxins has been done with aflatoxins, and some methods have also been tested for fumonisin decontamination. Ammoniation, which is effective for detoxification of aflatoxins (Park, 1993), reduced the concentration of detectable fumonisins in maize (Chourasia, 2001; Norred *et al.*, 1991; Park *et al.*, 1992), but failed to reduce toxicity (Norred *et al.*, 1991). Detoxification by a modified nixtamalisation procedure (Park *et al.*, 1996), exposure to ozone (McKenzie *et al.*, 1997), or by irradiation (Ferreira-Castro *et al.*, 2007) has also been investigated. To our knowledge, no technology has been established for large scale, routine application.

The reaction of fumonisins with reducing sugars at elevated temperatures (Murphy *et al.*, 1996) may be considered an intermediate between a decontamination process and fumonisin-consuming food or feed processing, because the reaction also takes place in non-enzymatic browning. However, a practical application of the reaction for fumonisin detoxification is not straightforward. With 1 M fructose (Lu *et al.*, 1997) or 100 mM glucose (Liu *et al.*, 2001), FB_1 had still not completely reacted after 48 h of incubation at 80 °C. Lu *et al.* (2002) studied the reaction rates with a range of reducing sugars, at different temperatures, and over a range of initial

D-glucose and FB_1 concentrations, and then calculated the reaction kinetics. The same authors found that in a non-enzymatic browning maize model system with 100 mM D-glucose added to FB_1 contaminated ground maize, eight days of incubation at 60°C were required to decrease FB_1 to half of the initial concentration, and at 80 °C, only two days were needed but addition of alpha-amylase was required to prevent gelatinisation of the maize starch (Lu *et al.*, 2002). No significant amounts of FB_1-sugar adducts are formed in the course of making and frying tortilla chips (Voss *et al.*, 2001), and corn meal or masa contain almost no reducing sugars for such a reaction to occur. However, addition of sugar, especially glucose, to maize grits before extrusion or to muffins before baking helped to reduce FB_1 levels (Castelo *et al.*, 2001), and the reaction was enhanced when a higher extrusion temperature was used (Seefelder *et al.*, 2001).

Enzyme-catalysed biotransformation

Since mycotoxins are natural toxins, biodegradation is an attractive approach for removing them from food or feed (Karlovsky, 1999). Black yeast strains (Duvick *et al.*, 1998) and bacterial strains (Benedetti *et al.*, 2006; Heinl *et al.*, 2010) that can catabolise fumonisins were isolated, and genes for fumonisin degradation from the black yeast strains *Exophiala spinifera* and *Rhinocladiella atrovirens* (Blackwell *et al.*, 1999), from the gram-negative bacterium designated isolate 2412.1 or ATCC 55552 (GenBank AR301774, US patent 6538177), and from *Sphingopyxis* sp. MTA144 (Heinl *et al.*, 2010) were identified and sequenced. The black yeast strains share the first step of fumonisin catabolism with the bacterial strains: a carboxylesterase releases the two TCA side chains by hydrolytic cleavage, so that FB_1 is converted to HFB_1. For the further metabolism of HFB_1, the yeast strains use an amine oxidase, which requires molecular oxygen and produces H_2O_2 as a by-product of the reaction (Blackwell *et al.*, 1999). The same reaction product, 2-keto-HFB_1, is produced by the bacterial strains via a different enzymatic mechanism, catalysed by an aminotransferase (Heinl *et al.*, 2010, 2011). The reaction requires a co-substrate as amino group acceptor, for instance pyruvate, which is converted to alanine. No genes or enzymes for the further catabolism of fumonisins in the black yeast strains have become known, but for bacterium ATCC 55552 and *Sphingopyxis* sp. MTA144, complete gene clusters also encoding enzymes for the proposed further downstream catabolic pathway are available (GenBank entries AR301774 and FJ426269). A possible application of genes from fumonisin catabolism pathways for generation of transgenic plants and fumonisin detoxification *in planta* has been considered (Duvick, 2001), but to our knowledge, no studies on the application of enzymes for fumonisin detoxification in food or feed processing, or as feed enzymes for activity in the gastrointestinal tract, have been reported.

4. Toxicity of fumonisin-derived reaction products

Fumonisin conjugates formed by thermal processing

While there is no doubt that thermal treatment at sufficiently high temperature in the course of food processing can reduce the amount of extractable and detectable fumonisins in maize matrix, the fate of the fumonisins, the reaction products, and their toxicities are not fully understood. Some loss of fumonisins may be accounted for by incomplete extraction from processed maize food (Scott and Lawrence, 1994). As already mentioned above, fumonisins were shown to undergo Maillard reaction between the 2-amino group and reducing sugars in the course of thermal processing of maize, but only to a small extent that has been judged insufficient to account for the reduction in detectable fumonisins (Seefelder *et al.*, 2001). The predominant reaction of fumonisins during thermal processing of maize seems to be TCA-mediated coupling to plant matrix (Kim *et al.*, 2003; Meister, 2001; Shier, 2000; Shier and Abbas, 1999). Seefelder *et al.* (2003) used a model system and showed that fumonisins can bind via their TCA moieties to the carboxyl group of saccharides, the epsilon-amino group of lysine, or the thiol group of cysteine. Evidence for the relevance of this reaction and binding to both starch and proteins, distinguished by extraction with a sodium dodecyl sulphate solution, comes from the finding that considerable amounts of HFB_1 can be released from corn flakes and other processed maize products by alkaline hydrolysis (Kim *et al.*, 2003; Park *et al.*, 2004; Shier and Abbas, 1999). However, alkaline hydrolysis can release HFB_1 in excess of the amount that is extractable and detectable as FB_1 also from samples of raw maize, where matrix binding was concluded to be non-covalent (Dall'Asta *et al.*, 2009, 2010). To our knowledge, TCA-mediated conjugates of FB_1 have not been isolated and their toxicity is unknown. It has been proposed that the conjugates that might be generated from bound fumonisins in the course of digestion, such as FB_1-lysine, FB_1-cysteine and FB_1-glucose, could have much higher bioavailability than FB_1 itself (Shier, 2000).

Even though the thermally catalysed Maillard reaction between the 2-amino group of fumonisins and reducing sugars seems to be of minor importance in normal processing of maize for food or feed, and a detoxification step that is based on the reaction may be hard to implement, the reaction products were studied extensively. The structures of initial, intermediate and final products of the reaction were determined (Howard *et al.*, 1998; Lu *et al.*, 2002; Poling *et al.*, 2002). Absorption of products of the reaction between FB_1 and reducing sugars from the gastrointestinal tract of rats was found to be higher than absorption of FB_1 (Dantzer *et al.*, 1999; Hopmans *et al.*, 1997), but the conjugates showed no hepatocarcinogenicity or hepatotoxicity in rats (Liu *et al.*, 2001; Lu *et al.*, 1997).

Fumonisin-glucose reaction products were non-toxic, contrary to the equimolar amount of unreacted fumonisins, when fed to swine (Fernandez-Surumay *et al.*, 2005), and the reaction products were only toxic for swine when they were given intraperitoneally at very high dose, but not at a lower dose (Fernandez-Surumay *et al.*, 2004). The purified reaction product N-carboxymethyl-FB_1 was also found to be non-toxic for mice (Howard *et al.*, 2002) and much less toxic than the parent compound in a toxicity assay with brine shrimp (Hartl and Humpf, 2000). Another conjugate to the 2-amino group of FB_1, N-acetyl-FB_1, is also known to have reduced toxicity (Abbas *et al.*, 1993; Gelderblom *et al.*, 1993; Howard *et al.*, 2002) and not to block ceramide synthase (Norred *et al.*, 1997), and a free amino group is thought to play a key role in toxicity. However, ceramide synthase inhibition by N-acetyl-FB_1 has also been published (Van der Westhuizen *et al.*, 1998). Since N-acetyl-FB_1 was later found to be unstable and rearrange to ceramide synthase-inhibiting O-acetylated derivatives of FB_1 (Norred *et al.*, 2001), the previously observed effects may not actually have been caused by N-acetyl-FB_1, but by spontaneously formed impurities. Derivatisation of FB_1 by diazotisation of the amino group was also found to reduce toxicity (Lemke *et al.*, 2001).

Hydrolysed fumonisins

Since fumonisins can be hydrolysed both by a well-established and traditional food processing method, nixtamalisation, and by specific microbial enzymes (Duvick *et al.*, 1998; Heinl *et al.*, 2010), HFB_1 and the hydrolysis products of other fumonisins can be considered very important metabolites. The toxicity of hydrolysed fumonisins has been studied extensively, all the more because experimental evidence from early work was hard to reconcile. In rat feeding trials with purified HFB_1, the toxicity was greatly reduced compared to the parent substance (Gelderblom *et al.*, 1993), but when nixtamalised fungal culture material was fed, the reduction of toxicity was not as strong (Hendrich *et al.*, 1993; Voss *et al.*, 1996, 1998). Even though in many *in vitro* assays HFB_1 was found to be less toxic than FB_1 (Abbas *et al.*, 1995; Flynn *et al.*, 1996, 1997; Hartl and Humpf, 2000; Lamprecht *et al.*, 1994; Norred *et al.*, 1992, 1997; Schmelz *et al.*, 1998; Van der Westhuizen *et al.*, 1998), some experimental results showed that toxicity was similar to or higher than the parent compound (Abbas *et al.*, 1993; Gelderblom *et al.*, 1993). A possible explanation for the seeming discrepancy of findings may be based on the fact that even though FB_1 is a much stronger inhibitor of ceramide synthase, HFB_1 also has affinity to the enzyme and will interfere with sphingolipid biosynthesis if the concentration is high enough (Humpf *et al.*, 1998; Merrill *et al.*, 1993; Norred *et al.*, 1997; Schmelz *et al.*, 1998). Since absorption of HFB_1 from the rat intestinal tract is almost as poor as absorption of FB_1 and both substances are mostly excreted in faeces (Dantzer *et al.*, 1999; Hopmans *et al.*, 1997), a concentration of HFB_1 that will produce a response of toxicity markers can be reached much more easily *in vitro* than by feeding. Acylation of HFB_1, catalysed by ceramide synthase (Humpf *et al.*, 1998), may also contribute to higher *in vitro* toxicity of hydrolysed fumonisins, compared to *in vivo* toxicity. N-acyl HFB_1 is a stronger ceramide synthase inhibitor and more toxic than HFB_1 (Abou-Karam *et al.*, 2004; Hartl and Humpf, 2000; Humpf *et al.*, 1998). It was shown to form also *in vivo* in rats when high HFB_1 concentrations were present after intraperitoneal dosing (Seiferlein *et al.*, 2007). However, after oral uptake the concentration of HFB_1 in blood or tissues is low (Dantzer *et al.*, 1999), and therefore the amount of N-acyl HFB_1 that can be formed may be too low to significantly affect the oral toxicity of HFB_1. More recently, feeding trials with mice (Howard *et al.*, 2002) and rats (Collins *et al.*, 2006) with purified HFB_1, and an experiment where HFB_1 was given intraperitoneally to pregnant mice (Voss *et al.*, 2009), confirmed that after hydrolysis, the interference with sphingolipid metabolism and toxicity was reduced, compared to the parent substance. The embryotoxic effect of fumonisins, especially their potential to elicit neural tube defect, was also found to be reduced or absent after hydrolysis when FB_1 and HFB_1 were tested *in vitro* (Flynn *et al.*, 1996, 1997) and *in vivo* (Collins *et al.*, 2006; Voss *et al.*, 2009). The early experiments with alkaline treated fungal culture material were also repeated more recently, and nixtamalisation reduced disturbance of sphingolipid metabolism and toxicity, especially when maize matrix was present with the culture material (Burns *et al.*, 2008). Compared with pure HFB_1, fungal culture material treated with base is less well defined, and nixtamalisation typically causes only partial but not complete hydrolysis of fumonisins. The nixtamalised culture materials used in the early trials were analysed and found to be free of intact fumonisins, but alkaline extraction for analysis (Voss *et al.*, 1996) may have contributed to hydrolysis, and from the reports it is not clear if the materials were free of partially hydrolysed fumonisins. With one exception, where an insensitive system was used (Caloni *et al.*, 2002), we are not aware of toxicity data for partially hydrolysed fumonisins, but it can be assumed that with one of the two tricarballylic acid side chains retained, they should have the same affinity to ceramide synthase and similar toxicity as the complete molecules. Therefore, partially hydrolysed fumonisins may have been responsible for some of the effects that were observed in the early feeding trials with nixtamalised *Fusarium* culture material.

Mechanisms of fumonisin toxicity other than ceramide synthase inhibition cannot be ruled out (Abel and Gelderblom, 1998; Gelderblom *et al.*, 2001; Gutiérrez-Nájera *et al.*, 2005; Theumer *et al.*, 2008; Wattenberg *et al.*, 1996; Yin *et al.*, 1996), but interference with sphingolipid metabolism can be considered the main mechanism (Merrill *et al.*, 2001; Riley *et al.*, 2001). Based on the reduced inhibition

of ceramide synthase activity, we think that the available experimental evidence is sufficient to consider hydrolysis of fumonisins in food or feed as a detoxification step.

5. Opportunities for the application of enzymes

The first enzyme of the known fumonisin catabolism pathways from both eukaryotic and prokaryotic microorganisms is a carboxylesterase, which releases TCA side chains from fumonisins by hydrolytic cleavage (Duvick *et al.*, 1998; Heinl *et al.*, 2010). Based on the evidence for reduction of toxicity by hydrolysis of fumonisins, as discussed above, fumonisin carboxylesterase could be suitable for fumonisin detoxification by enzymatic transformation, and work on the fumonisin carboxylesterase encoded by the *fumD* gene of *Sphingopyxis* sp. MTA144 (Heinl *et al.*, 2010) is in progress. The enzyme lends itself to a technological application because as a hydrolase, it requires no co-factors. However, enzymes which are active further downstream in the catabolic pathways may also need to be considered if fumonisins cannot be sufficiently detoxified by hydrolysis alone. The 2-amino group of fumonisins, which is critical for toxicity, can be removed from HFB_1 by an amine oxidase from *E. spinifera* (Blackwell *et al.*, 1999), or by specific bacterial aminotransferases (Heinl *et al.*, 2010, 2011). Technological applications of these enzymes may not be as straightforward, because amine oxidases require molecular oxygen, which is scarce in the gastrointestinal tract and many food processing steps, and aminotransferases require co-substrates such as alpha-keto acids, which serve as amino group acceptors. Co-substrates would need to be provided with the enzyme, unless they were naturally present in the matrix. However, the HFB_1 aminotransferase FumI of *Sphingopyxis* sp. MTA144 has recently been produced and purified as a recombinant enzyme, so that the reaction characteristics and kinetics can be determined and the application potential can be evaluated (Hartinger *et al.*, 2010). The natural enzymes may not perform best in a technological application, because of their inherent properties such as pH and temperature optima, but enzymes can be tuned to specific reaction conditions by enzyme engineering.

Prospects for enzymatic detoxification of feed

A general prerequisite for the activity of enzymes is water, because enzyme and substrate need to meet in solution. Water is involved in many food processing steps, but when maize is used for animal feed, there is not necessarily any wet processing. However, one possibility to enzymatically catalyse reactions in food or feed is in the gastrointestinal tract. It is the site where enzymes, either from the organism itself or from its native intestinal microbial community, are responsible for digestion. The use of several enzymes as feed additives has been established to enhance digestion and the nutritional value of feed. Phytases, which release phosphate from phytate, a phosphate storage molecule of plants which is therefore naturally contained in feed, are probably the best known example (Lei and Porres, 2003). The enzymes make phosphate from phytate bioavailable for absorption, which helps to save inorganic phosphate that is otherwise required as a feed additive. They also provide an environmental benefit because phosphate contained as phytate is stripped from manure. In the same way, application of enzymes for fumonisin detoxification also seems feasible. However, the technological challenge of delivering sufficient enzymatic activity to the intestinal tract, so that the reaction is completed before the fumonisins are absorbed, remains to be met.

Pigs are known to be quite sensitive to fumonisins (Grenier *et al.*, 2011; Haschek *et al.*, 2001; Voss *et al.*, 2007), and although many modern pig farms are equipped for dry feeding, the more traditional liquid feeding can potentially provide nutritional advantages and more palatable feed. Modern, computer-operated systems are designed to prevent possible hygiene problems, and soaking of feed in water or whey, or controlled fermentation, could be suitable for the application of fumonisin detoxification enzymes. Another feed processing step, which has grown enormously in importance because of the increased demand for fuel ethanol, is conversion of maize to DDGS by generation and fermentation of maize mash. While fumonisins normally accumulate in DDGS (Bothast *et al.*, 1992), the fermentation could provide the aqueous solution, time and temperature required for fumonisin detoxification enzyme activity.

Prospects for enzymatic detoxification of food

If enzymes can be applied for detoxification of fumonisins contained in feed, the results could also be used to evaluate the safety of the technology for a possible future application in human nutrition. In the subsistence farming population that is most exposed to fumonisins, the focus should certainly remain on the best possible practices for growing, storing and preparing food, even if fumonisin detoxification enzymes become an option. The use of a specific clay mineral for the reduction of aflatoxin exposure in a subsistence farming population has already been attempted (Afriyie-Gyawu *et al.*, 2008; Wang *et al.*, 2008), but an enzyme formulation that has to be taken separately from food, or used in the course of food preparation, may not be widely accepted. However, from a technological standpoint, enzymatic treatment of food for fumonisin transformation should be possible, both in home cooking and in industrial food processing. A limitation is that any thermal steps in food processing would not be suitable for the application of enzymes. When maize, which is the most significant source of fumonisins not only in animal but also in human nutrition, is washed before preparation and consumption, an enzyme that is added to the wash water could transform fumonisins. The dose of enzyme

would have to be sufficient to complete the reaction in a rather short incubation period, unless a washing or soaking step can be extended, and the wash water would have to be in a suitable temperature range. In terms of fumonisin detoxification, nixtamalisation is probably the most important food processing method, because fumonisins are hydrolysed under the alkaline conditions during cooking in lime water, and because in Latin America, nixtamalisation is a traditional and widely used process that also enhances the nutritional value of maize. However, extraction of fumonisins and conversion to the less toxic hydrolysed fumonisins is usually incomplete. In the course of the actual alkaline treatment, enzymatic support of fumonisin hydrolysis may not be possible because of the high pH, but the nixtamal is washed before it is ground to masa, and the washing steps may be the point at which an enzyme could be applied to complete hydrolysis and extraction. Another possible opportunity for enzymatic treatment would be in the processing of wet masa, before it is baked into tortillas. Generally, when maize meal is mixed with water in the course of food preparation, enzymatic transformation of fumonisins may be possible. Detoxification enzymes could also be applied in the course of ethanol fermentation of traditional Xhosa maize beer in South Africa (Shephard *et al*., 2005) or Chicha in South America. Fumonisin B_2 can be present in grape juice (Logrieco *et al*., 2009) and red wine (Mogensen *et al*., 2010), where it could also be enzymatically degraded during fermentation.

Enzymatic fumonisin transformation and toxicity

The toxicity of reaction products is a key concern when enzymatic fumonisin transformation is considered. As discussed above, experimental evidence suggests that the reaction products of fumonisin carboxylesterases, hydrolysed fumonisins, should be less toxic than the parent molecules. However, in the course of technological development of a carboxylesterase for fumonisin detoxification, confirmation of whether hydrolysis provides a sufficient reduction of toxicity will be necessary. Since hydrolysed fumonisins also have, albeit reduced, affinity to ceramide synthase, and since they can be acylated by ceramide synthase (Humpf *et al*., 1998), there may be a practical fumonisin concentration limit up to which detoxification by hydrolysis is feasible. Furthermore, the sensitivity to hydrolysed fumonisins may vary between different animal species and humans. To our knowledge, the toxicity of 2-keto-HFB_1, the reaction product of yeast amine oxidases or bacterial aminotransferases for deamination of HFB_1, has not yet been tested. It seems reasonable to speculate that the toxicity of 2-keto-HFB_1 should be very low, because the amino group is known to play an important role in the toxicity of FB_1, and because 2-keto-HFB_1 obviously cannot be acylated by ceramide synthase like HFB_1.

For an application of enzymes in food or feed, the issue of conjugated fumonisins must also be considered. Conjugates formed by TCA-mediated linkage to matrix are present not only in thermally processed, but also in unprocessed maize (Dall'Asta *et al.*, 2009, 2010). The effect of conjugation on the toxicity of fumonisins is not known. Matrix binding may play a key role in fumonisin toxicity, because when macromolecules with TCA-coupled fumonisins are digested in the intestinal tract, certain proposed conjugates such as fumonisyl-lysine, fumonisyl-cysteine or fumonisyl-glucose (Seefelder *et al.*, 2003) could be released. Such conjugates might be actively absorbed from the intestinal tract and could therefore have much higher bioavailability than free fumonisins (Shier, 2000). Enzymatic hydrolysis of such conjugates, catalysed by a fumonisin carboxylesterase, could therefore greatly reduce toxicity. On the other hand, matrix binding of fumonisins might also reduce their bioavailability, especially if they are coupled to indigestible macromolecules. In that case, enzymatic release of fumonisins from such conjugates, catalysed by a fumonisin carboxylesterase, could cause an increase in bioavailable fumonisins, albeit in hydrolysed form. The considerations become even more speculative when enzymatic release of the TCA chains before baking or cooking in the course of food preparation is contemplated. TCA-mediated thermal coupling of fumonisins to matrix will be precluded if the side chains are removed before heating, but it is not clear whether the formation of potentially highly bioavailable conjugates or a fumonisin-sequestering reaction would be prevented.

The N-conjugates of fumonisins are also important for the potential use of the technology. Fumonisin derivatives with a blocked 2-amino group are not considered toxic, but hydrolytic cleavage of the side chains might make them toxic. At least the N-acyl HFB_1 molecules with various lengths of the acyl chains were described as potent inhibitors of ceramide synthase (Abou-Karam *et al.*, 2004; Humpf *et al.*, 1998). N-conjugates of fumonisins are not known to occur naturally in maize in significant amounts and their formation in the course of thermal processing seems to be negligible (Voss *et al*., 2001). However, the range of mycotoxin conjugates that can be formed by plants is remarkable (Berthiller *et al.*, 2006), and some conjugated fumonisins might still be awaiting discovery.

6. Conclusions

Various methods for fumonisin decontamination have been explored, but apart from physical separation, none of these methods could be implemented in routine processing of maize. Using established methods of food processing, fumonisins can only be reduced in detectable concentration, but not completely eliminated, and the nature, bioavailability and toxicity of some of the reaction products is still unknown. For these reasons, enzymatic

transformation of fumonisins may be a favourable contribution to support animal health and consumer safety. The fumonisin transforming enzymes that have become known, especially the fumonisin carboxylesterases, may be suitable for such an application. For detoxification of animal feed, which is often provided dry and not processed through any steps that would allow enzymatic treatment, an enzyme could be provided as a feed additive for activity in the gastrointestinal tract. Enzymatic treatment of maize mash, when DDGS for use as animal feed is generated in the course of ethanol fermentation, would also be feasible. Application of enzymatic fumonisin detoxification in animal nutrition could help to establish the safety of the technology, if a subsequent application for treatment of food is desired. Any wet food processing steps, such as washing of maize or nixtamal, or mixing of dough, could potentially be suitable for enzymatic treatment. The specific characteristics of enzymes, such as co-factor requirement, pH and temperature optima have to be considered. The toxicity of reaction products is not yet sufficiently established, and several questions associated with possible carboxylesterase-catalysed release of hydrolysed fumonisins from matrix-conjugates, as well as other issues with fumonisin-matrix interactions, remain to be addressed.

Specific enzymes for fumonisin biotransformation seem to have the potential for technological application. However, they can only be one contribution to the many different approaches, including plant breeding and agriculture, feed and food processing, nutrition, toxicological studies and biotechnology, which will still be required, together with the consideration of social aspects, before the fumonisins can be considered a problem of the past.

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