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Enzyme characteristics of aminotransferase FumI of *Sphingopyxis* sp. MTA144 for deamination of hydrolyzed fumonisin B_1

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Abstract Fumonisins are carcinogenic mycotoxins that are frequently found as natural contaminants in maize from warm climate regions around the world. The aminotransferase FumI is encoded as part of a gene cluster of *Sphingopyxis* sp. MTA144, which enables this bacterial strain to degrade fumonisin B_1 and related fumonisins. FumI catalyzes the deamination of the first intermediate of the catabolic pathway, hydrolyzed fumonisin B_1. We used a preparation of purified, His-tagged FumI, produced recombinantly in *Escherichia coli* in soluble form, for enzyme characterization. The structure of the reaction product was studied by NMR and identified as 2-keto hydrolyzed fumonisin B_1. Pyruvate was found to be the preferred co-substrate and amino group receptor (K_M=490 μM at 10 μM hydrolyzed fumonisin B_1) of FumI, but other α-keto acids were also accepted as co-substrates. Addition of the co-enzyme pyridoxal phosphate to the enzyme preparation enhanced activity, and saturation was already reached at the lowest tested concentration of 10 μM. The enzyme showed activity in the range of pH 6 to 10 with an optimum at pH 8.5, and in the range of 6°C to 50°C with an optimum at 35°C. The aminotransferase worked best at low salt concentration. FumI activity could be recovered after preincubation at pH 4.0 or higher, but not lower. The aminotransferase was denatured after preincubation at 60°C for 1 h, and the residual activity was also reduced after preincubation at lower temperatures. At optimum conditions, the kinetic parameters K_M=1.1 μM and k_{cat}=104/min were determined with 5 mM pyruvate as co-substrate. Based on the enzyme characteristics, a technological application of FumI, in combination with the fumonisin carboxylesterase FumD for hydrolysis of fumonisins, for deamination and detoxification of hydrolyzed fumonisins seems possible, if the enzyme properties are considered.

Keywords Mycotoxin · Detoxification · Degradation · Kinetics · Feed · Animal

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Introduction

Fumonisins are a group of structurally related mycotoxins produced by several species of *Fusarium* (Rheeder et al. 2002) as well as by *Alternaria alternata* (Chen et al. 1992), *Aspergillus niger* (Frisvad et al. 2007), and *Tolypocladium*

species (Mogensen et al. 2011). *Fusarium verticillioides* may be considered the most important fumonisin producer from the aspect of food and feed safety, as it is an important pathogen of maize in warm climate regions around the world. The fumonisins produced by this fungus were also the first to be isolated (Gelderblom et al. 1988; Bezuidenhout et al. 1988) and thoroughly studied (Marasas 2001). Fumonisin B_1 (FB_1), the most prevalent fumonisin of *F. verticillioides*, can cause equine leukoencephalomalacia (Marasas et al. 1988; Kellerman et al. 1990), porcine pulmonary edema (Harrison et al. 1990; Haschek et al. 2001), liver cancer in rats (Gelderblom et al. 1991), neural tube disorder in mice (Sadler et al. 2002; Gelineau-van Waes et al. 2005), alteration of the immune response in pigs (Taranu et al. 2005), and shows several other toxic effects in laboratory and domestic animals (Voss et al. 2007). Exposure to fumonisins is also associated with cancer and neural tube disorder in humans (Wild and Gong 2010). At the molecular level, fumonisins interfere with sphingolipid metabolism by inhibiting the enzyme ceramide synthase (Wang et al. 1991). The resulting imbalance of sphingolipids, which also have signaling functions as cellular messenger molecules, is responsible for toxic and carcinogenic effects of fumonisins (Merrill et al. 2001; Riley et al. 2001). However, other mechanisms of fumonisin toxicity and carcinogenicity were also proposed, including activation of mitogen-activated protein kinase (Wattenberg et al. 1996), lipid peroxidation (Abel and Gelderblom 1998), and alteration of the biosynthesis of other lipids in addition to the sphingolipids (Gelderblom et al. 2001).

The known fumonisin biodegradation pathways in black yeast strains (Blackwell et al. 1999) and bacteria (Duvick et al. 2003; Heinl et al. 2010) all start with the hydrolytic release of the two tricarballylic acid side chains, catalyzed by a fumonisin carboxylesterase. The second step is deamination of hydrolyzed fumonisin B_1 (HFB_1), either by an amine oxidase in black yeast strains (Blackwell et al. 1999) or an aminotransferase in bacterial strains (Heinl et al. 2010, 2011) (Fig. 1). Enzymatic detoxification of fumonisins may be a suitable approach to increase feed and food safety (Karlovsky 1999). However, it is unclear whether toxicity is sufficiently reduced by enzymatic cleavage of the two tricarballylic acid side chains. Hydrolyzed fumonisins were reported not to have cancer-initiating potency (Gelderblom et al. 1993), to be less hepatotoxic (Howard et al. 2002), less disruptive of sphingolipid metabolism, and not inducing neural tube disorder (Voss et al. 2009) in vivo. Hydrolysis of fumonisins by nixtamalization was reported to reduce, but not eliminate, toxicity (Hendrich et al. 1993; Voss et al. 1996). The in vitro effects of hydrolyzed fumonisins were reported to range from less (Flynn et al. 1997; Norred et al. 1997; Schmelz et al. 1998; van der Westhuizen et al. 1998; Seefelder et al. 2003) to more toxic than those of the intact fumonisins (Gelderblom et al. 1993). The 2-amino group of hydrolyzed fumonisins plays a key role for toxicity, since it can be acylated both in vitro (Humpf et al. 1998) and in vivo (Seiferlein et al. 2007), and the *N*-acyl-metabolites are even more cytotoxic than FB_1 (Humpf et al. 1998). In summary, deamination in addition to removal of the side chains may be necessary for complete fumonisin detoxification. Since the bacterial aminotransferases, contrary to the black yeast amine oxidases, do not require molecular oxygen for activity, they may be more suitable for applications in anaerobic environments, for instance as feed or food enzymes for detoxification directly in the intestinal tract of animals or humans. Another possible application would be in ethanol fermentation of maize mash, where fumonisins normally accumulate in the distillers dried grains and solubles, which are subsequently used as animal feed.

We have previously cloned the *fumI* gene of *Sphingopyxis* sp. MTA144, which catalyzes deamination of HFB_1 (Heinl et al. 2010), expressed the gene in *E. coli* and purified the enzyme (Hartinger et al. 2010). Here, we report the characterization of the enzyme together with its kinetic parameters as a starting point for evaluation of the application potential of the enzyme.

Materials and methods

Chemicals

FB_1 and ^{13}C-FB_1 were obtained from Biopure Referenzsubstanzen GmbH (Tulln, Austria). HFB_1 was prepared by complete hydrolysis of FB_1 with purified recombinant carboxylesterase FumD (Heinl et al. 2010). All other chemicals were purchased from Sigma (St. Louis, MO, USA) or Sigma–Aldrich (Steinheim, Germany). Water was purified by reverse osmosis in an arium® RO 61316 system (Sartorius Stedim Biotech GmbH, Göttingen, Germany).

Recombinant enzyme production and purification

Construction of the expression vector pET-30a-AT144HIS, gene expression in *E. coli* ArcticExpress(DE3), and purification of the 6xHis-tagged recombinant aminotransferase FumI by immobilized nickel affinity chromatography were previously described (Hartinger et al. 2010). The recombinant enzyme FumI-HIS comprises the FumI sequence (GenBank ACS27061) with a C-terminal extension of ASSVD KLAAALEHHHHHH. The calculated molecular mass is 48,271.5 Da. Preparations of FumI-HIS were stored at −20°C after addition of glycerol to 25%.

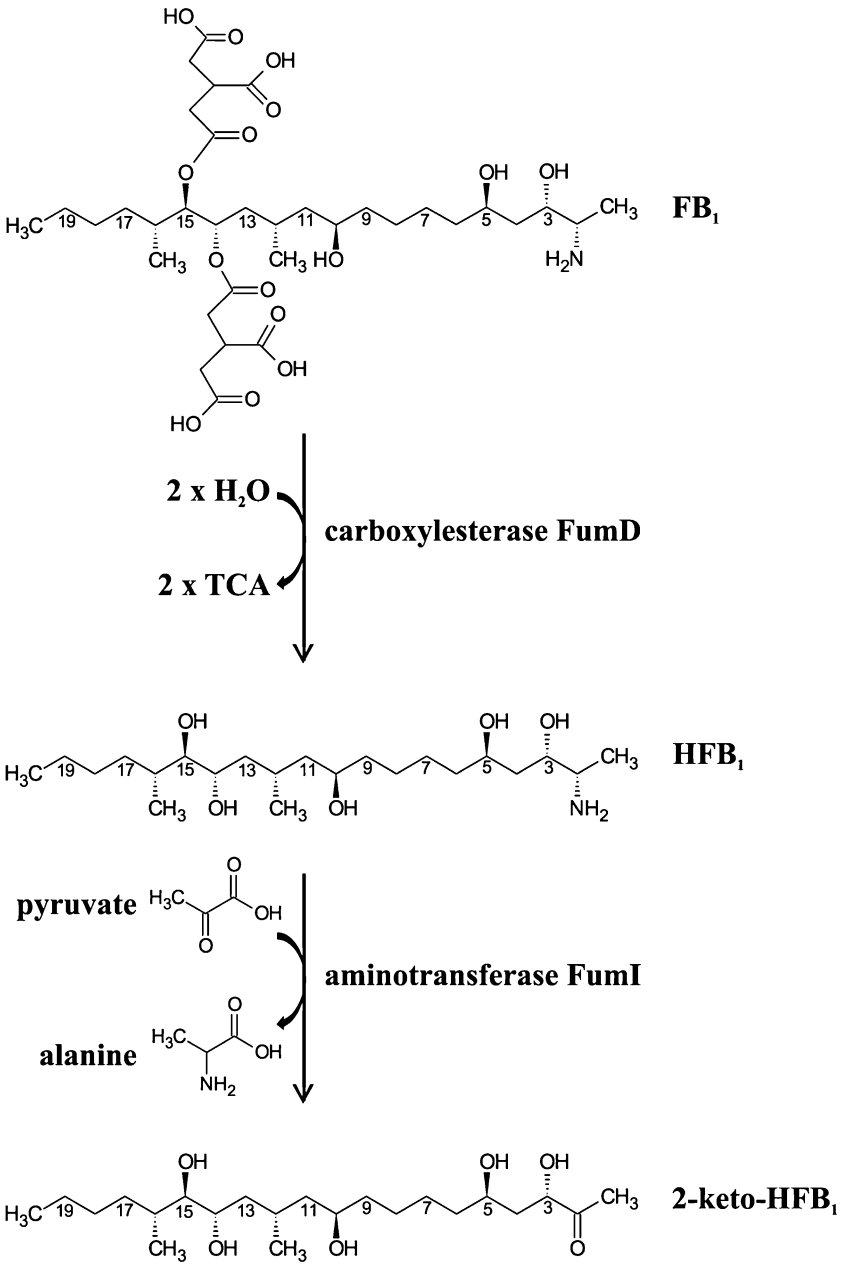

Fig. 1 Initial steps of fumonisin B_1-degradation pathway of *Sphingopyxis* sp. MTA144: FB_1 (2-amino-12,16-dimethyl-3,5,10-trihydroxy-14,15-propan-1,2,3-tricarboxyicosane) is substrate of the fumonisin carboxylesterase FumD, which catalyzes hydrolytic cleavage of both tricarballylic acid (TCA) chains off the core chain to produce HFB_1 (2-amino-12,16-dimethylicosane-3,5,10,14,15-pentol) and tricarballylic acid (1,2,3-propanetricarboxylic acid). Aminotransferase FumI transfers the 2-amino group from HFB_1 to pyruvate, producing 2-keto-HFB_1 (3,5,10,14,15-pentahydroxy-12,16-dimethylicosane-2-one) and alanine

Enzyme activity assays

Possible co-substrates of the aminotransferase were tested in a reaction system containing 94 ng/ml FumI-HIS in 20 mM Tris–HCl pH 7.4, 50 mM NaCl, 2 mM $CaCl_2$, 20 μM pyridoxal phosphate (PLP), and 0.1 mg/ml bovine serum albumin (BSA). HFB_1 as the substrate was employed at a concentration of 15 μM, and no co-substrate or 15 μM or 3 mM of the α-keto acids pyruvate, α-ketobutyrate, α-ketoglutarate, glyoxylate, or oxaloacetate, respectively, were added. Samples of 200 μl were taken immediately after addition of the enzyme to the reaction mixture (t=0 h), as well as after 0.5, 1, and 3 h of incubation in a water bath at 25°C. For correlation of enzyme activity with pyruvate and oxaloacetate concentration, the assay was performed as above except with 10 μM HFB_1, and co-substrate concentrations were varied from 0.01 to 100 mM. Aminotransferase activity was determined by calculating the slope of the initial, linear reaction rates in Δ μmol HFB_1 per liter per minute. Oxaloacetate was dissolved directly before use to avoid decarboxylation. To test the need for addition of PLP, the assay was performed as above with 15 μM HFB_1 and PLP concentrations ranging from 0 to 400 μM. The effect of salt on enzyme activity was tested by using 20 mM Tris–HCl pH 8.0 buffers with 1,000 mM, 100 mM, 10 mM NaCl, or without added NaCl, Teorell–Stenhagen buffer

pH 8.0 (Teorell and Stenhagen 1938), or 100 mM potassium phosphate buffer, all with 20 μM PLP, 0.1 mg/ml BSA, 3 mM pyruvate, and 15 μM HFB_1.

The optimum pH for enzyme activity was determined by setting Teorell–Stenhagen buffer to pH values ranging from pH 1.8 to 11.5 at 22°C (Teorell and Stenhagen 1938). The assay was performed using 67 ng/ml FumI-HIS, 10 μM HFB_1, 20 μM PLP, 0.1 mg/ml BSA, and 5 mM pyruvate at 25°C. Samples were taken before addition of enzyme and after 15, 30, 45, 60, 120, and 240 min. Enzyme activities were calculated from early time points, where reaction rates were linear, and are expressed as μM 2-keto-HFB_1 formed per minute. The optimum temperature for enzyme activity was determined by using the same conditions as for the pH dependence, with the buffer set to pH 8.0 at 22°C, and incubation temperatures in water baths ranging from 6°C to 50°C. Reaction mixtures were temperature-equilibrated for 30 min before addition of enzyme.

To determine pH stability of FumI-HIS, the Teorell–Stenhagen buffer was set to pH values between pH 1.1 and 10.7, and 14 μg/ml of enzyme was incubated in these buffers at 35°C for 1 h. The residual enzyme activity was determined in the same buffer at pH 7.8 after dilution of FumI-HIS to 50 ng/ml, using 15 μM HFB_1, 20 μM PLP, 0.1 mg/ml BSA, and 5 mM pyruvate. Samples were taken, and reaction rates were determined as for the correlation of activity with pH and temperature. The thermal stability of FumI-HIS was determined by incubating 14 μg/ml of enzyme in Teorell–Stenhagen buffer, set to pH 8.0 at 22°C, for 1 h in water baths at temperatures ranging from 7°C to 60°C. Residual enzyme activity was measured at 35°C after dilution in the same buffer with final concentrations of FumI-HIS, HFB_1, PLP, BSA, and pyruvate as described for pH stability. Samples were taken, and reaction rates were calculated as for the determination of optimum pH and temperature.

Enzyme kinetics were determined by incubating 10 ng/ml FumI-HIS at 35°C in 20 mM Tris–HCl pH 8.0, 20 μM PLP, 0.1 mg/ml BSA, and 5 mM pyruvate, while the concentration of HFB_1 was varied from 0.1 to 100 μM. Samples were taken, and reaction rates were determined as above.

Liquid chromatography–mass spectrometry

Samples taken from enzyme assays were inactivated at 99°C for 10 min and stored frozen. After thawing, mixing, and centrifugation, aliquots of samples containing nominally 0.2 nmol HFB_1, but no more than 100 μl, were dried at 50°C under a stream of nitrogen and dissolved in 200 μl solvent with ^{13}C-FB_1 as internal standard. Thus, the final nominal HFB_1 concentration for analysis was 1 μM, or less if the original concentration was less than 2 μM. All samples were analyzed using the previously described instrumental setup and method (Heinl et al. 2010), to which 2-keto-HFB_1 has been integrated (Hartinger et al. 2010).

Preparative isolation of 2-keto-HFB_1 and structure determination by NMR

FumI-HIS was produced in *E. coli* ArcticExpress (DE3) as previously described (Hartinger et al. 2010). Biomass was resuspended in 1/100 of the culture volume, homogenized in a French Press, and 1.2 ml of the clarified lysate was added to 4.05 mg HFB_1 in 25 ml buffer (20 mM Tris–HCl pH 8.0, 6.6 mM pyruvate, 30 μM PLP, and 0.1 mg/ml BSA). After incubation at 30°C, complete deamination of HFB_1 was confirmed by liquid chromatography–mass spectrometry (LC–MS). The reaction mixture was centrifuged, lyophilized, and dissolved in 6 ml of 40% acetonitrile in water (*v*/*v*). After centrifugation, 600-μl portions were separated on a preparative Agilent 1100 HPLC system (Agilent, Waldbronn, Germany) consisting of a G1361A pump, a G2260A autosampler, an adjustable flow splitter (Analytical Scientific Instruments, CA, USA), an evaporative light-scattering detector (SEDEX LT-ELSD Model 85, Sedere, Alfortville, France), a G1365B multi-wavelength detector, and a G1364B fraction collector. A Gemini-NX C18 column (150×21.2 mm, 5-μm particle size, 110-Å pore size; Phenomenex, Aschaffenburg, Germany) protected by a Gemini C18 pre-column (15×21.2 mm) was operated at a flow rate of 16 ml/min using gradient elution. Mobile phase A, consisting of acetonitrile/H_2O (10:90, *v*/*v*), was degassed by membrane filtration. Acetonitrile was used as mobile phase B. The gradient was as follows: 0–0.2 min: 0% B, 0.2–5 min: linear increase to 95% B, 5–6.4 min: isocratic elution at 95% B, 6.4–6.5 min: linear decrease to 0% B, 6.5–9.5 min: re-equilibration at 0% B. The column effluent was split 1:70, one part moving into the evaporative light-scattering detector and the main part passing through the multivariate wavelength detector to the fraction collector. The 2-keto-HFB_1 eluted between 4.85 and 5.7 min and fractions were collected in that time window. The combined fractions were evaporated to dryness on a rotary evaporator, dissolved in 7 ml of ethyl acetate, and transferred into an 8-ml screw cap vial; the ethyl acetate solution was evaporated to dryness under a stream of nitrogen at 50°C. The 2-keto-HFB_1, a viscous, clear, and colorless oily substance, was stored at 4°C until NMR analysis.

^1H- and ^{13}C-NMR spectra were obtained from CD_3CN solutions using an Avance DRX-400 FT-NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany), operating at 400.13 MHz for ^1H and 100.62 MHz for ^{13}C, at 295 K using a 5-mm inverse broadband *Z*-gradient probe head. Data were recorded and evaluated using TOPSPIN 1.3

software (Bruker Biospin). All pulse programs were taken from the Bruker software library. Chemical shifts were established on the basis of residual solvent resonances.

Results

Biochemical characterization of FumI-HIS

Clarified cell lysates of *E. coli* overexpressing the *fumI* gene were previously used to obtain preliminary information, namely that HFB_1 is a substrate and that an α-keto acid such as pyruvate is required as co-substrate for the activity of the aminotransferase FumI (Heinl et al. 2010). The detailed biochemical characterization reported here was made using FumI-HIS, which was prepared and purified to electrophoretic homogeneity as described before (Hartinger et al. 2010). This enzyme preparation was found to be stable for several months when stored in 25% glycerol at −20°C. Clarified cell lysates of *E. coli* ArcticExpress (DE3) producing FumI and FumI-HIS from the same expression vector pET-30a under identical conditions showed a very similar HFB_1 deamination activity, indicating that the His-tag does not affect the enzymatic activity.

The α-keto acids pyruvate, α-ketobutyrate, α-ketoglutarate, glyoxylate, and oxaloacetate were tested as potential co-substrates for aminotransferase FumI (Fig. 2). No deamination of HFB_1 was detectable when no co-substrate was added or in the presence of α-ketoglutarate, while pyruvate, oxaloacetate, glyoxylate, and α-ketobutyrate, when included at 3 mM concentration, supported the reaction, with HFB_1 deamination efficiency decreasing in this order. When tested with 10 μM HFB_1, the enzyme showed maximal activity at 10 mM pyruvate, and the K_M for pyruvate was 490 μM. Deamination activity was significantly reduced when the concentration of the co-substrate pyruvate was in the same range as that of HFB_1, and substrate inhibition was observed for pyruvate concentrations in the range of 50–100 mM (Fig. 3). With oxaloacetate as co-substrate, reaction rates were lower, and the K_M for oxaloacetate was 4,150 μM (Fig. 3). Addition of PLP enhanced the aminotransferase activity of FumI-HIS, and saturation was reached already at the lowest concentration tested (10 μM PLP) since a further increase in PLP concentration to 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 300, and 400 μM in the assay mixture did not result in a further increase in the reaction rate. Without added PLP, FumI-HIS showed about 23% of the maximum activity.

Addition of NaCl to the 20 mM Tris–HCl (pH 8.0) reaction buffer reduced the HFB_1 deamination rate (Fig. 4). When using 100 mM potassium phosphate or Teorell–Stenhagen buffer, enzyme activity was also lower than in 20-mM Tris–HCl buffer of the same pH. Nevertheless, Teorell–Stenhagen buffer was used to determine the optimum pH for enzyme activity, since this buffer can be adjusted to any pH between 2.0 and 12.0 (Teorell and Stenhagen 1938). Aminotransferase FumI-HIS was active in the range of pH 6.5 to 9.7 and showed a rather sharp optimum at pH 8.0 and 8.5 (Fig. 5a). The enzyme was active over a broad temperature range of 6°C to 45°C with an optimum for the 30 min assay at 35°C (Fig. 5b). Significant and rapid thermal inactivation was observed at 50°C, where traces of 2-keto-HFB_1 were formed only at the

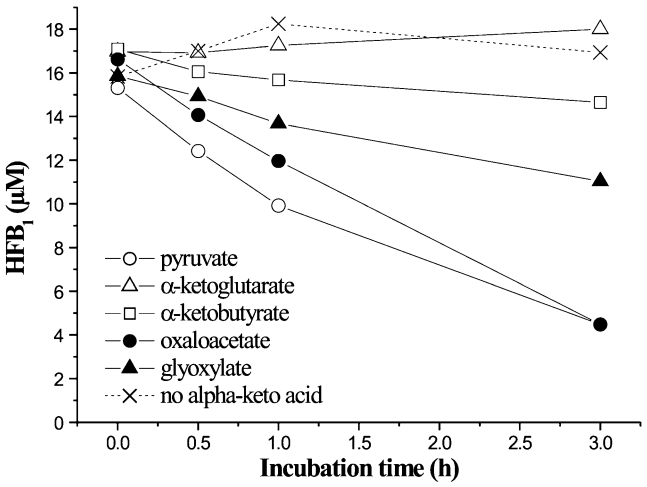

Fig. 2 Comparison of HFB_1 deamination activity of FumI-HIS with five different α-keto acids as co-substrate. The reactions were incubated in 20 mM Tris–HCl buffer (pH 7.4) at 25°C with 94 ng/ml FumI and 3 mM α-keto acid

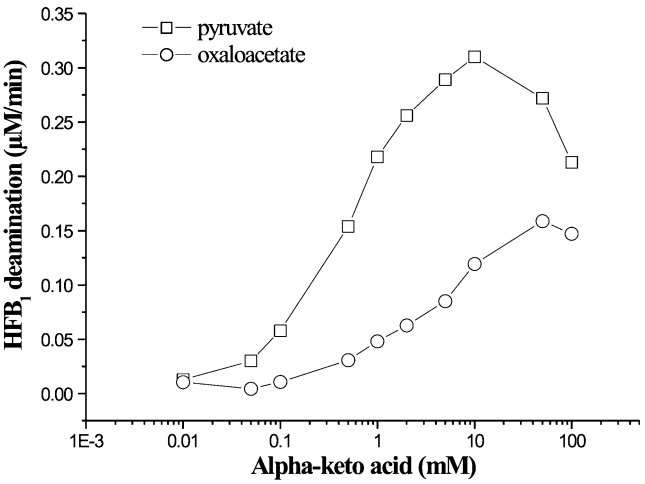

Fig. 3 Correlation between pyruvate concentration or oxaloacetate concentration and HFB_1 deamination activity. The reactions were incubated with 94 ng/ml FumI-HIS and 10 μM HFB_1 in 20 mM Tris–HCl (pH 7.4) with 0.1 mg/ml BSA, 20 μM PLP, and the indicated concentration of pyruvate or oxaloacetate at 25°C. Samples were taken and analyzed, and reaction rates were calculated as described in Materials and Methods. The reaction mixtures without co-substrates (negative controls), which are not shown in the semi-logarithmic plot, gave HFB_1 deamination rates of zero

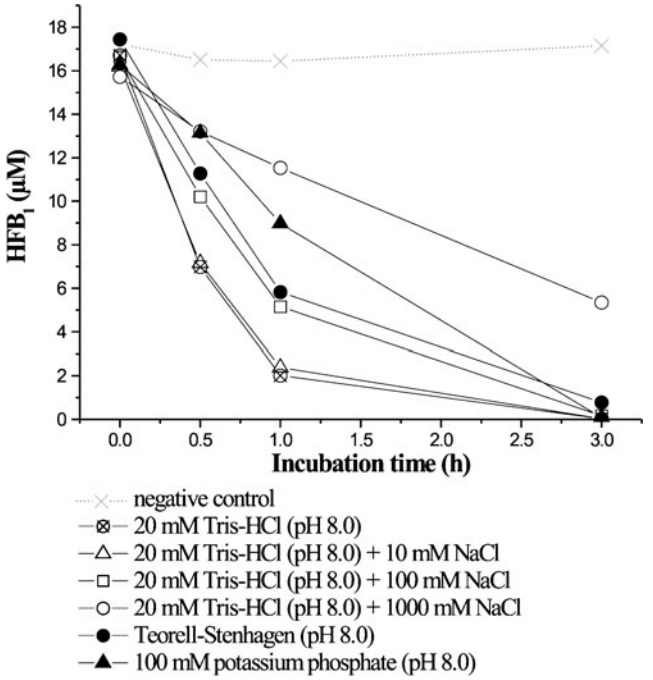

Fig. 4 Effect of different buffers and salt concentrations on the HFB_1-deamination rate of aminotransferase FumI-HIS. Negative control: 20 mM Tris–HCl (pH 8.0) with buffer instead of enzyme

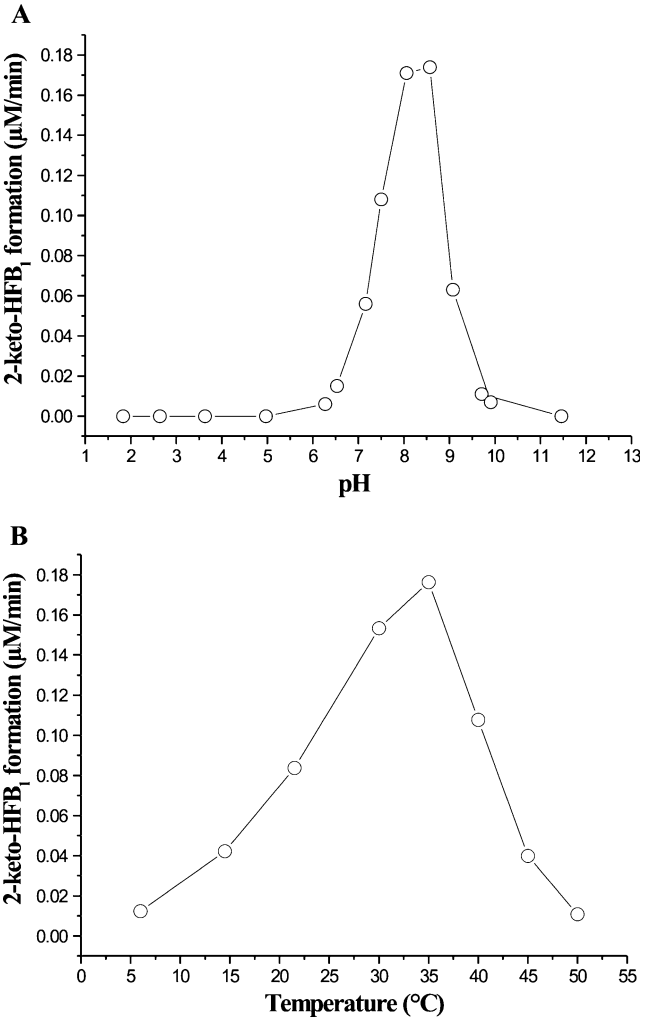

Fig. 5 Determination of optimum pH (**a**) and temperature (**b**) for FumI-HIS activity. The reactions were incubated with 67 ng/ml FumI-HIS and 10 μM HFB_1 in Teorell–Stenhagen buffer, with supplements as described in Materials and Methods, set to the indicated pH values (**a**) or to pH 8.0 (**b**), at 25°C (**a**) or the indicated temperatures (**b**)

beginning of the incubation period (data not shown). Chromatograms of samples from the HFB_1 deamination reaction of this experiment at 35°C are shown in Fig. 6. Furthermore, FumI-HIS was incubated with intact FB_1 instead of hydrolyzed FB_1 under identical conditions, but FB_1 was not deaminated. Reaction with a hydrolyzed total fumonisin extract from *F. verticillioides* culture material performed under the same conditions showed that, in addition to HFB_1, HFB_2, and HFB_3 were substrates of FumI-HIS as well. When FumI-HIS was preincubated at an acidic pH and 35°C for 1 h before HFB_1 deamination was measured, the activity was reduced after incubation at pH 6.5 or less, and completely abolished after incubation at pH below 4.0 (Fig. 7a). Incubation at pH 8.0 for 1 h at various temperatures resulted in a steady decrease of FumI activity with increasing temperature, and the enzyme was completely inactivated after incubation at 60°C. Even after incubation at 7°C, a 15% loss of enzyme activity compared to non-preincubated FumI was determined (Fig. 7b).

The apparent steady-state kinetic parameters of FumI-HIS catalyzed HFB_1 deamination were determined for a saturating co-substrate concentration of 5 mM pyruvate in 20 mM Tris–HCl buffer (pH 8.0) at 35°C, and the Michaelis–Menten plot is shown in Fig. 8. The apparent V_{max} value of 0.022 μM/min was reached at 5 μM HFB_1 when 10 ng/ml of FumI-HIS was present in the reaction mixture. A further increase in substrate (HFB_1) concentration resulted in a significant reduction of the reaction rate, indicating substrate inhibition. The k_{cat} value was calculated to be 104 min^{-1} using the theoretical molecular mass of FumI-HIS, and the Michaelis constant K_M for HFB_1 was determined to be 1.1 μM. The specific HFB_1 deamination activity was 2.2 μmol/min/mg.

Structure determination of the reaction product by NMR

From the 1D ^1H- to ^{13}C-APT-NMR spectra, it was obvious that the sample did not contain a single species, but three different forms. In order to analyze the molecular structures of these compounds, $^1H^1H$ COSY, $^1H^{13}C$ HSQC, and $^1H^{13}C$ HMBC spectra were recorded. Using these two-dimensional methods, all of the structures could be elucidated unambiguously, and complete assignments of ^1H and ^{13}C signals were established (Table 1). Thus, the structures were

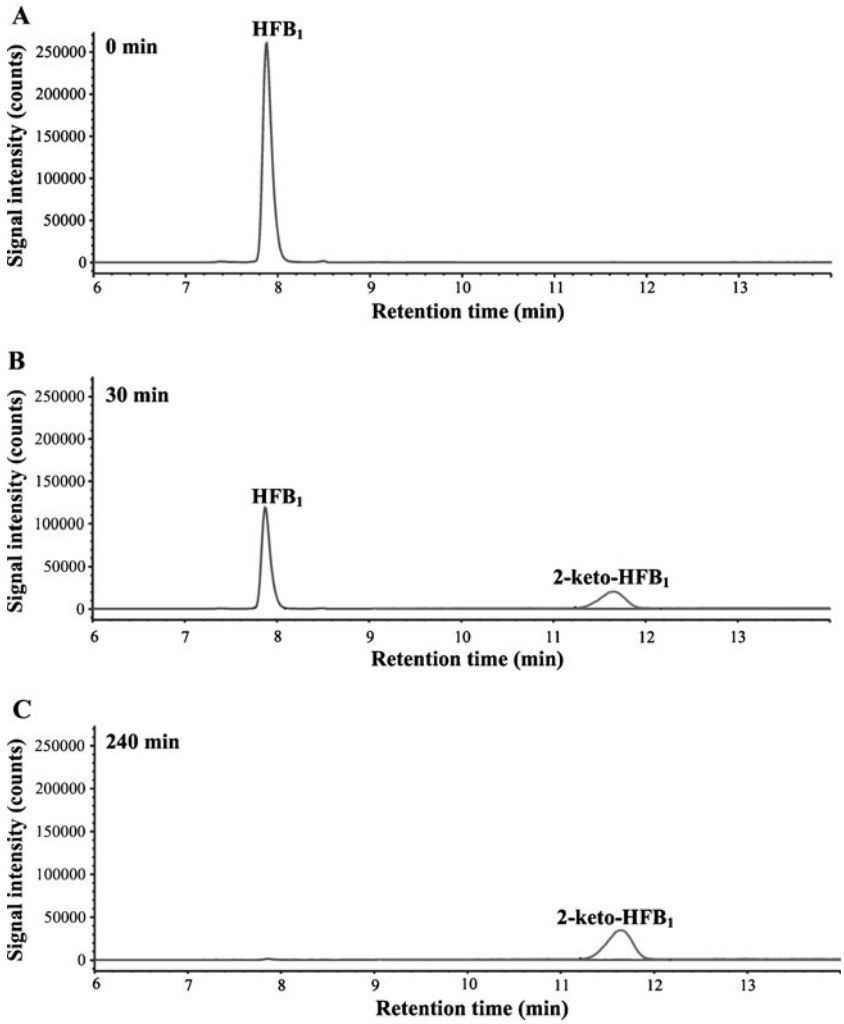

Fig. 6 LC–MS chromatograms of samples from a HFB_1 transamination reaction. Samples of 10 μM HFB_1 in Teorell–Stenhagen buffer (pH 8.0) at 35°C were taken before (**a**), 30 min (**b**), or 240 min (**c**) after addition of purified aminotransferase FumI-HIS to a final concentration of 67 ng/ml. HFB_1 and 2-keto-HFB_1 were separated on a C8 reversed phase column in a formate–acetonitrile gradient and detected in SIM mode

identified as 2-keto-HFB_1 and two cyclic hemiketals formed by ring closure between the 2-keto moiety and the 5-OH. While the hemiketals have already been described (Blackwell et al. 1999), the open-chain keto form has not yet been characterized.

Discussion

Gastrointestinal detoxification of fumonisins by specific enzymes is a promising concept to ameliorate the effects that fumonisins, which are frequently found as natural contaminants of maize in large parts of the world, have on the health and performance of domestic animals. It is important to know the characteristics of an enzyme-catalyzed reaction before a technological application can be considered. We determined some key properties of the aminotransferase FumI of *Sphingopyxis* sp. MTA144 with respect to its reaction with fumonisins. The enzyme is active with hydrolyzed FB_1, the first intermediate in all known pathways of FB_1 catabolism, and not with FB_1 itself and catalyzes the transfer of the 2-amino group onto a suitable acceptor (Heinl et al. 2010). Since the corresponding amino group of structurally similar sphinganine is acylated by ceramide synthase, which is the molecular target that is inhibited by fumonisins (Wang et al. 1991), the deamination reaction can be considered as important for detoxification of fumonisins. To our knowledge, no reports on the toxicity of 2-keto-HFB_1 have been published, and we have not studied its toxicity yet. However, the amino group is thought to play a key role for the toxicity of FB_1 (Gelderblom et al. 1993; Lu et al. 1997; Norred et al. 2001; Fernandez-Surumay et al. 2004), and obviously, 2-keto-HFB_1 cannot be acylated in a similar way as HFB_1 (Humpf et al. 1998; Seiferlein et al. 2007). Therefore, it seems reasonable to speculate that 2-keto-HFB_1 may be non-toxic.

Numerous aminotransferases have been identified and studied since the first description of this class of enzymes (Needham 1930), and several are used in industrial applications for the production of a range of natural and non-natural amino acids and amines (Taylor et al. 1998;

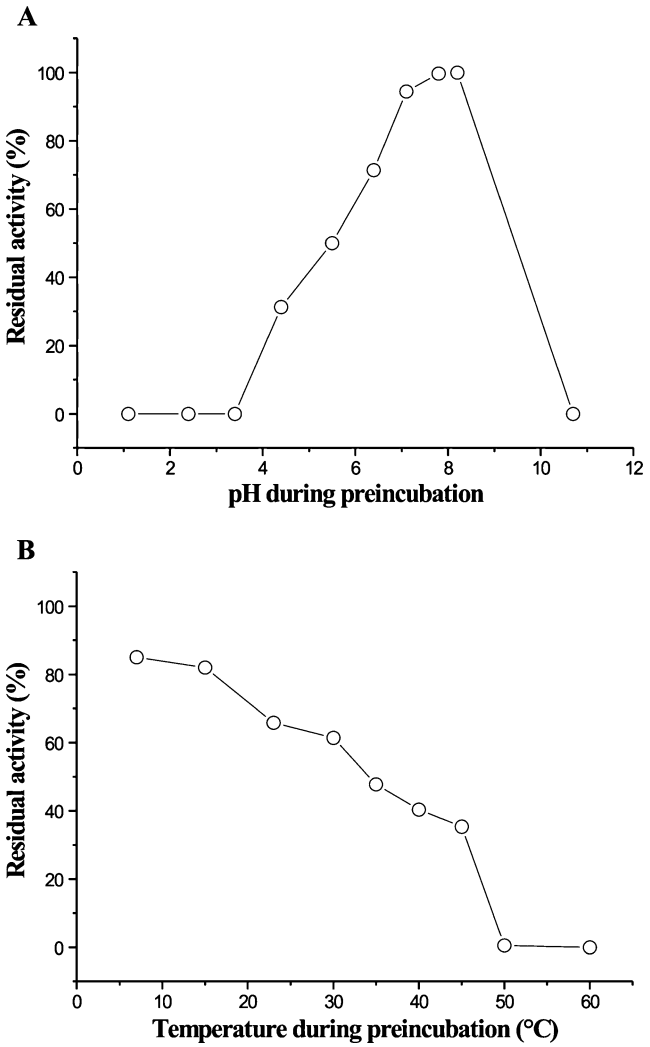

Fig. 7 Stability of FumI-HIS at various pH values (**a**) and temperatures (**b**). Fourteen micrograms per milliliter FumI-HIS was incubated in Teorell–Stenhagen buffer at the indicated pH at 35°C (**a**) or in the same buffer at pH 8.0 at the indicated temperature (**b**) for 1 h. Residual enzyme activity was determined as described in Materials and Methods. The measured activities after incubation at pH 8.0 (**a**) or without preincubation (**b**) were set to 100%

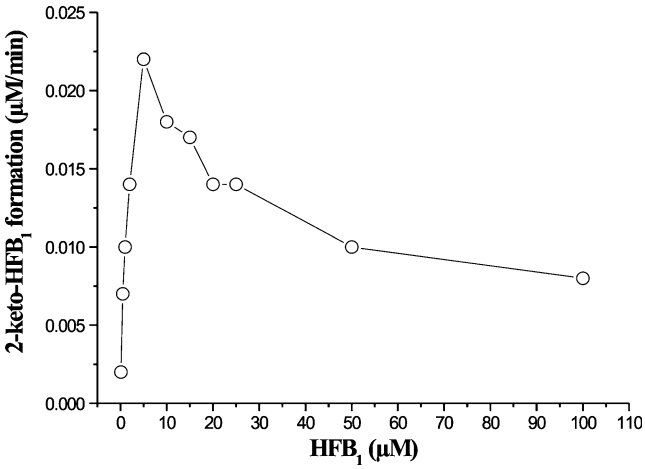

Fig. 8 Initial HFB_1 deamination reaction rate as a function of substrate concentration. The reactions were performed with 10 ng/ml FumI-HIS and 5 mM pyruvate, a saturating co-substrate concentration, at 35°C in 20 mM Tris–HCl pH 8.0, 20 μM PLP, 0.1 mg/ml BSA. HFB_1 concentrations ranged from 0.1 to 100 μM

Hwang et al. 2005; Zhu and Hua 2009). However, this is the first characterization of an aminotransferase that catalyzes deamination of a fumonisin. Our work may lead to the further expansion of the range of aminotransferase applications to mycotoxin detoxification. Since FB_1 is not a substrate for FumI, a possible technological application of the enzyme will require its combined use together with a fumonisin carboxylesterase such as FumD, so that HFB_1, the substrate of FumI, is generated by hydrolytic cleavage of the two tricarballylic acid side chains of FB_1. In this respect, the substrate specificity of FumI is quite remarkable, since the direct vicinity of the 2-amino group is identical in FB_1 and HFB_1 (Fig. 1). Since HFB_2 and HFB_3 are also deaminated by the enzyme, the 10-hydroxyl and 5-hydroxyl groups of HFB_1, which are lacking in HFB_2 and HFB_3, respectively, are apparently not essential for substrate recognition. The ability of FumI to use several different α-keto acids as amino group acceptors is also noteworthy. Even though pyruvate was the preferred co-substrate when various α-keto acids were tested at the identical molar concentrations in our study, it is tempting to speculate that the relaxed co-substrate specificity of FumI may have biological significance for its function in *Sphingopyxis* sp. MTA144. Depending on the available α-keto acids, FumI could thus produce alanine from pyruvate, aspartate from oxaloacetate, or glycine from glyoxylate in vivo. The affinity of FumI for its co-substrate (Fig. 3), which is much lower than the affinity for HFB_1 (Fig. 8) when judged by the K_M values, may also be important for the enzyme in vivo, since the compatible α-keto acids are also intermediates in energy metabolism. In this context, it is interesting to note that the *fum* gene cluster of *Sphingopyxis* sp. MTA144 encodes three genes, the tricarballylate proton symport pump FumG, the tricarballylate dehydrogenase FumE, and the citrate utilization protein B FumF, which supposedly allow utilization of the two tricarballylic acid side chains of FB_1 and their conversion to citric acid (Heinl et al. 2010). Oxaloacetate and glyoxylate, which both accept the amino group during the FumI-catalyzed reaction, can both be generated from citric acid in the Krebs cycle or the glyoxylate cycle. For a technological application of FumI, this co-substrate requirement together with the comparatively low K_M value implies that pyruvate or one of the other suitable α-keto acids will have to be present or will have to be provided together with the enzyme at

Table 1 ^{1}H and ^{13}C NMR assignment of 2-keto-HFB_1 and its hemiketal forms

	2-Keto-HFB_1		α-Hemiketal[a]		β-Hemiketal[a]	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	2.14	25.8	1.32	22.8	1.29	25.0
2	–	212.4	–	106.9	–	102.2
3	4.28	75.7	3.87	78.2	3.80	76.8
4	1.72, 1.50	41.4	2.40, 1.35	40.5	2.23, 1.52	38.9
5	3.70	68.7	3.97	77.0	3.80	76.1
6	1.50–1.40	38.8	1.58, 1.44	37.4	1.45–1.40	38.5
7	1.45–1.25	≈27	1.45–1.25	≈27	1.45–1.25	≈27
8	1.45–1.25	≈27	1.45–1.25	≈27	1.45–1.25	≈27
9	1.40–1.35	39.4	1.40–1.35	39.4	1.40–1.35	39.4
10	3.59	69.8	3.59	69.8	3.59	69.8
11	1.48, 1.06	44.4	1.48, 1.06	44.4	1.48, 1.06	44.4
12	1.87	26.9	1.87	26.9	1.87	26.9
12-Me	0.94	21.7	0.94	21.7	0.94	21.7
13	1.40–1.25	40.1	1.40–1.25	40.1	1.40–1.25	40.1
14	3.61	70.2	3.61	70.2	3.61	70.2
15	3.16	80.2	3.16	80.2	3.16	80.2
16	1.55	35.6	1.55	35.6	1.55	35.6
16-Me	0.84	16.4	0.84	16.4	0.84	16.4
17	1.61, 1.20	32.2	1.61, 1.20	32.2	1.61, 1.20	32.2
18	1.36, 1.20	30.0	1.36, 1.20	30.0	1.36, 1.20	30.0
19	1.25–1.35	23.9	1.25–1.35	23.9	1.25–1.35	23.9
20	0.90	14.5	0.90	14.5	0.90	14.5

[a] α/β according to Blackwell et al. (1999)

millimolar concentration to enable efficient and fast HFB_1 deamination. However, if the enzyme is to be used for gastrointestinal HFB_1 deamination, this co-substrate requirement might be covered to some extent by the presence of α-keto acids in the chymus. It cannot be excluded that other amino group acceptors that we have not yet identified may enable higher reaction rates even at low concentration.

Blackwell et al. have noted before that 2-keto-HFB_1 can undergo cyclization between the 5-hydroxyl group and C-2 of 2-keto-HFB_1 to form two isomers of a hemiketal, and that this reaction is favored by the presence of methanol in the solvent (Blackwell et al. 1999). We made the same observation and adapted our procedure for purification and NMR characterization of 2-keto-HFB_1 to work without methanol. Nevertheless, the NMR characterization showed that our preparation of 2-keto-HFB_1 was not pure, and the signals indicative of the hemiketals were still present. As the C-1–C-5 part of 2-keto-HFB_1 resembles a simplified ketose structure, an equilibrium between the open-chain and cyclized forms may be the explanation for this behavior since in carbohydrate chemistry, such equilibria are ubiquitous. This may also be one reason that the 2-keto-HFB_1 peaks that eluted from a C8 HPLC column were broader than the HFB_1 peaks.

Overexpression of FumI in *E. coli* and the subsequent purification procedure (Hartinger et al. 2010) result in enzyme preparations that apparently are not saturated with the prosthetic group PLP, as can be concluded from the finding that addition of PLP to such an enzyme preparation enhances activity. Aminotransferases were reported to occur as mixtures of their apo- and holo-forms also when isolated from their natural environment (Moss 1976). Increasing the PLP concentrations above 10 μm in the reconstitution experiments of recombinant FumI-HIS showed no further increase of aminotransferase activity, which is consistent with the tight binding of PLP that has been reported for other aminotransferases (Ford et al. 1980; Eliot and Kirsch 2004). If batches of enzyme need to be prepared for technological application in the future, they can be saturated with low concentrations of PLP before their application to ensure that FumI is predominantly available as a holoenzyme.

FumI-HIS displayed HFB_1 deamination activity in all buffers we tested, and these covered a wide range of salt concentrations (Fig. 4). This indicates that the proposed application of FumI is unlikely to be limited by buffer salt requirements. The lack of enzyme activity at acidic pH (Fig. 5a) rules out that FumI is already active in the stomach when provided as an animal feed supplement.

However, FumI can be expected to function in sections of the upper intestine where the pH is close to neutral or even slightly higher. The temperature optimum of 35°C and good activity at 40°C found for FumI-HIS (Fig. 5b) imply that the actual body temperature of animals, which e.g. was reported to be 38.8°C for piglets (Ingram and Legge 1970), should be well suited for high enzyme activity. Based on our determination of pH stability (Fig. 7a), it will be necessary to provide FumI in a formulation that enables shuttling through the stomach without inactivation at low pH. Simultaneously, the enzyme could thus be protected from proteolytic degradation. Technologies for microencapsulation and targeted release in the intestine have previously been developed (Champagne and Fustier 2007).

We were surprised to see a reduction of HFB_1 deamination activity after preincubation of FumI-HIS at 7°C, because we had observed that a preparation of enzyme that was stored in the fridge apparently retained full activity over several weeks. Maybe handling of the enzyme at low concentration contributed to the observed loss of activity. Likewise, the maximal specific reaction rate measured in the experiment shown in Fig. 8, using 10 ng/ml FumI-HIS, was lower than in the experiment shown in Fig. 3, when 94 ng/ml FumI-HIS was used.

The K_M value we determined for HFB_1 deamination, 1.1 μM, corresponds to 0.794 mg/kg FB_1. Since the fumonisin concentrations frequently found in maize (Binder et al. 2007; Gonzalez Pereyra et al. 2008; Gong et al. 2009; Monbaliu et al. 2010) and known to have toxic effects on animals (Ross et al. 1991; Taranu et al. 2005) are higher, FumI should have sufficient affinity for the substrate HFB_1 to enable detoxification in the range of fumonisin concentrations that are relevant in agriculture. The decrease of FumI activity observed at HFB_1 concentrations higher than 10 μM, which is equivalent to 7.22 mg/l FB_1, can be interpreted as substrate inhibition. Since the guidance value for the maximum acceptable concentration of FB_1 and FB_2 in e.g. pig feed is 5 mg/kg according to the European Commission Recommendation 2006/576/EC, this observed inhibition of activity at higher HFB_1 concentrations may not be relevant from a technological point of view. In case enzymatic detoxification by a combination of FumD and FumI of a highly contaminated commodity is required, substrate inhibition of FumI may be avoided by adapting FumD activity to that of FumI, so that the HFB_1 is continuously formed in sub-inhibitory concentrations.

Feed enzymes may be useful for the degradation of antinutritive substances that are naturally contained in agricultural commodities, or to enhance the nutritional value of feed. The use of phytase as a feed enzyme to release phosphate from phytate that is naturally contained in plant material is well established (Lei and Porres 2003; Rao et al. 2009). Based on the enzyme characteristics reported here, FumI may also be suitable for future applications as a feed enzyme to enhance the salubriousness of maize that is naturally contaminated with fumonisins, if the specific properties and requirements of FumI are considered in this application.

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Conflict of interest The authors declare no conflict of interest.

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