(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
30 August 2012 (30.08.2012)

WIPO | PCT

(10) International Publication Number
WO 2012/113827 A1

(51) International Patent Classification:
A23K 1/165 (2006.01) *C12N 9/16* (2006.01)

(21) International Application Number:
PCT/EP2012/053009

(22) International Filing Date:
22 February 2012 (22.02.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
11155883.9 24 February 2011 (24.02.2011) EP

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2012/113827 A1

(54) **Title**: FEED PROCESSING ENZYMES

(57) **Abstract**: The invention relates to a feed supplement for treating a zearalenone (ZON) contaminated feed product comprising a recombinant Gl stable serine hydrolase enzyme, feed products containing such supplement, methods of preparing a grain based feed product and the use of a recombinant serine hydrolase for *in situ* detoxification of a mycotoxin upon feeding.

FEED PROCESSING ENZYMES

FIELD OF THE INVENTION

The present invention relates to ZON lactonase enzymes (ZONASES) for degrading mycotoxins.

BACKGROUND OF THE INVENTION

Several plant pathogenic and/or post-harvest Fusarium species on cereals produce toxic substances of considerable concern to livestock and poultry producers, e.g., deoxynivalenol, T-2 toxin, HT-2 toxin, diacetoxyscirpenol and zearalenone (ZON). Zearalenone is found worldwide in a number of cereal crops, such as maize, barley, oats, wheat, rye, rice, millet and sorghum. Zearalenone production does not seem to occur in significant amounts prior to harvest, but under proper environmental conditions, it is readily produced on corn and small grains in storage.

Mycotoxin formation may occur when the causative fungi grow on crops in the field, at harvest, in storage, or during feed processing; essentially whenever favorable conditions for their formation prevail. Generalizations about geographical distribution of particular types of mycotoxins are difficult due to widespread distribution of the causative fungi. However, aflatoxins and fumonisin tend to prevail in warmer climates, while cooler regions with higher moisture are subject to ochratoxin, zearalenone, vomitoxin (deoxynivalenol, DON), T2 toxin, and others. Each mycotoxin has its own particular effect, and all can be devastating. Co-contamination by one or more types of mycotoxin occurs naturally, and exerts a greater negative impact on health and productivity of livestock than contamination by individual mycotoxins.

Zearalenone is heat-stable, and it is not destroyed by long storage, roasting, or by the addition of propionic acid or mold retardants. Despite their structural dissimilarity to the steriodal estrogens, zearalenone and several of its derivatives possess estrogenic activity. Zearalenone undergoes a folding such that hydroxyl or potential hydroxyl groups become appropriately orientated to facilitate binding to tissue receptors that normally bind estrogens.

Zearalenone is the primary toxin causing infertility, abortion or other breeding problems, especially in swine. The symptoms are especially severe in prepubertal gilts including enlarged mammae, swelling of uterus and vulva, and atrophy of the ovaries. In severe cases, prolapse of the vulva and rectum may occur. Boars exhibit enlarged mammae and atrophied testes. The mycotoxin is present in the meat from animals feeding on contaminated grain as well as in bread baked from contaminated wheat. While cases of poisoning of humans are rare there is concern about the effect of the long term exposure of humans to such an estrogenic activity.

Microbial enzymes are a resource for many biotechnological applications. Microbial strains or their enzymes may be used for degradative (bioremediation) or synthetic (biotransformation) purposes. Modern bioremediation or biotransformation

strategies may even involve microbial catalysts or strains designed by protein engineering or pathway engineering. Prerequisite for developing such modern tools of biotechnology is a comprehensive understanding of microbial metabolic pathways, of the structure and function of enzymes, and of the molecular mechanisms of biocatalysis.

The *in vitro* inactivation of mycotoxins by cultivating the substrate with selected enzymes in a liquid, slurry or paste, using epoxidase, lactonase, laccase or cutinase is disclosed in WO9612414, WO2009077447 and WO2009080701. Takahashi-Ando et al. (Biochem. J. 2002; 364: 1-6) describe a lactonohydrolase for the detoxification of zearalenone at a pH optimum of pH 9-10. At low pH (< pH 4.5) the enzyme was unstable and irreversibly inactivated.

A method of removing mycotoxins from animal feed is described in WO99053772 whereby a modified yeast cell walls extract and a mineral clay is added to the feed product.

A feed additive based on dried yeast and sea algae is provided by Biomin GmbH (Herzogenburg, Austria) for deactivating mycotoxins.

Takahashi-Ando et al. (Appl Environ Microbiol. 2004 Jun;70(6):3239-45) discloses the detoxification with recombinant E.coli and transgenic rice cells at pH 6.2.

An esterase is disclosed in WO0123581 which is similar to the triacylglycerol hydrolase.

There is still a need for further means of detoxifying animal food products. It is thus the objective of the invention to provide for improved enzymatic detoxification of mycotoxins in feed products.

SUMMARY OF THE INVENTION

The inventors of the present invention have discovered that ZON in a food product can be degraded by treating the food product with zonase from the serine hydrolase superfamily that is surprisingly GI stable to enable *in situ* mycotoxin deactivation in or upon passage of the gastrointestinal tract.

Accordingly, in a first aspect the invention provides for a food supplement for treating a ZON contaminated feed product comprising a recombinant GI stable zonase.

The feed supplement according to the invention preferably contains the enzyme in the purified form, with a purity of at least 70%, preferably of at least 80% and most preferred of at least 90%.

The feed supplement according to the invention preferably contains a BTA-1 hydrolase or a BTA-2 hydrolase

Specifically the enzyme as used according to the invention has a pH stability in the range of pH 3 to 8 and a protease stability in the presence of pancreatic enzymes, e.g. is GI stable. Thus, the enzyme is stable under conditions comparable to passage of the stomach (acidic) and small intestine (neutral). The enzyme advantageously is

stable against pepsin and pancreatin digestion, essentially maintaining its ZON detoxification activity. The enzyme advantageously is active at low pH, preferably in the range of pH 3 to 7.

The enzyme preferably has ZON detoxification activity as measured in a cell based assay, specifically using a standard proliferation assay to detect the decrease of ZON estrogenic activity with the human breast adenocarcinoma cell line MCF-7.

A further aspect of the invention is the use of a zonaxe for *in vitro* or *in situ* detoxification of a mycotoxin.

Zonase for use as an active ingredient or in a combination enzyme product are selected from the group consisting of hydrolases, lipases, esterases and depolymerases.

Preferred zonases are selected from the group consisting of BTA-hydrolase, in particular BTA-1 hydrolase or BTA-2 hydrolase, lipase and PBS A depolymerase. Specifically the following enzymes are preferred: BTA hydrolase 2 of *Thermobifida fusca*, BTA hydrolase 1 of *Thermobifida fusca*, lipase of *Streptomyces exfoliatus*, PBS A depolymerase of *Acidovorax delafieldii*, and triacylglycerol hydrolases of *Streptomyces sp.*, or variants thereof.

In a preferred embodiment according to the invention a variant of BTA-2 hydrolase is used with a sequence identity of at least 50%, preferably at least 60%, or at least 70%, or at least 80% or at least 90% to the parent BTA-2 hydrolase.

Therefore, a GI stable BTA-2 hydrolase variant with ZON detoxification activity is provided according to the invention, having a sequence identity of at least 50%, preferably at least 60%, or at least 70%, or at least 80% or at least 90% compared to the parent BTA-2 hydrolase.

A further aspect of the invention is a GI stable BTA-2 hydrolase variant with ZON detoxification activity according to the invention, having at least 25 consecutive amino acids selected from the amino acid sequence as set forth in SEQ ID NO: 6 and at least 25 consecutive amino acids selected from the amino acid sequence as set forth in SEQ ID NO: 10 or SEQ ID NO: 8.

A further aspect of the invention is a GI stable BTA-2 hydrolase variant with ZON detoxification activity according to the invention, having at least 50 consecutive amino acids selected from the amino acid sequence as set forth in SEQ ID NO: 6 and at least 25 consecutive amino acids selected from the amino acid sequence as set forth in SEQ ID NO: 10 or SEQ ID NO: 8.

Another aspect of the invention is a GI stable BTA-2 hydrolase variant with ZON detoxification activity according to the invention, having at least 25 consecutive amino acids selected from the amino acid sequence as set forth in SEQ ID NO: 6 and at least 50 consecutive amino acids selected from the amino acid sequence as set forth in SEQ ID NO: 10 or SEQ ID NO: 8.

Specifically preferred is a BTA-2 hydrolase variant selected from the group consisting of SEQ ID NO: 13, 14, 15 and 16.

According to the invention there is further provided a food product comprising grain and at least one recombinant GI stable serine hydrolase enzyme, such as those used in the food supplement according to the invention.

The food product according to the invention preferably contains the enzyme in a dry mixture, e.g. as a premixed animal feed product or in a kit of parts containing (a) the grain product and (b) the feed supplement, which may be admixed to the grain product in the desired ratio. Preferably the grain is selected from corn, wheat, barley, rye, rice, sorghum and millet.

According to the invention there is further provided a method of preparing a grain based food product, wherein the grain is treated with a recombinant GI stable serine hydrolase enzyme, such as used in the food supplement according to the invention. The food product may be treated *in vitro* or *in situ*, e.g. in the process of feeding and/or digesting, to effectively detoxify eventually present mycotoxins *in vivo*.

It is preferred that the enzyme is added to the grain in a dry mixture to obtain a premixed product commercialized as a ready-to-use mixture, or readily prepared before feeding.

Thus, according to the invention there is further provided a new use for a GI stable recombinant zonase for *in situ* detoxification of a mycotoxin upon feeding.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the detoxification of zearalenone by cleavage of the lactone ring and subsequent decarboxylation.

Figure 2 shows the zearalenone degrading activity of the parental enzymes.

Figure 3 shows the zearalenone degrading activity of the purified hits. The zearalenone degrading activity of the hits is comparable with the activity of the parental enzymes.

Figure 4 Sequence alignment of parentals and hits

Figure 5 shows the nucleic acid sequence of triacylglycerol hydrolase (Myco 021)

Figure 6 shows the amino acid seqence of triacylglycerol hydrolase (Myco 021)

Figure 7 shows the nucleic acid sequence of zearalenone hydrolase (Myco 022)

Figure 8 shows the amino acid sequence of zearalenone hydrolase (Myco 022)

Figure 9 shows the nucleic acid sequence BTA-hydrolase 2 (Myco 023)

Figure 10 shows the amino acid sequence of BTA-hydrolase 2 (Myco 023)

Figure 11 shows the nucleic acid sequence lipase (Myco 024)

Figure 12 shows the amino acid sequence of lipase (Myco 024)

Figure 13 shows the nucleic acid sequence PBS A depolymerase (Myco 025)

Figure 14 shows the amino acid sequence of PBS A depolymerase (Myco 025)

Figure 15 shows the nucleic acid sequence BTA-hydrolase 1 (Myco 026)
Figure 16 shows the amino acid sequence of BTA-hydrolase 1 (Myco 026)
Figure 17 shows the amino acid sequence of Myco H03
Figure 18 shows the amino acid sequence of Myco H04
Figure 19 shows the amino acid sequence of Myco H10
Figure 20 shows the amino acid sequence of Myco H11

DETAILED DESCRIPTION OF THE INVENTION

In the context of this invention the term "zearalenone" or ZON comprises the mycotoxin zearalenone produced from certain *Fusarium* sp. The IUPAC name is (4S, 72E)-15,17-dihydroxy-4-methyl-3-oxabicyclo[12.4.0]octadeca-12,15,17,19-tetraene-2,8-dione. The term "zearalenone" also comprises any derivative of zearalenone which comprises an internal carboxylic ester bond susceptible for modification by a cutinase or a hydrolase.

The term “zonase” means any enzyme which is capable of hydrolyzing the lactone ring of zearalenone (ZON) or of its derivatives. Preferably, the zonase is a serine hydrolase superfamily enzyme.

The term “GI stable” means the enzyme is stable under conditions comparable to passage of the stomach (acidic) and small intestine (neutral). Specifically the enzyme as used according to the invention has a pH stability in the range of pH 3 to 8 and a protease stability in the presence of gastric and pancreatic enzymes. Thus, the enzyme advantageously is stable against pepsin and pancreatin digestion, essentially maintaining its ZON detoxification activity.

As used herein, "pH stability" refers to the ability of an enzyme to retain its activity at a particular pH. According to the invention, an enzyme is pH stable if it retains 40% of residual activity after incubation for 30 min at pH 3.

The term "animal" denotes all animals, including human beings. Examples of animals are cattle, (including but not limited to cows and calves); mono-gastric animals, e.g. pigs or swine (including, but not limited to, piglets, growing pigs, and sows); poultry such as turkeys and chicken (including but not limited to broiler chicks, layers); and fish (including but not limited to salmon).

The term "food" or "food product" means any feed, compound, preparation, mixture, or composition suitable for, or intended for intake by an animal. The food product may be a product which apart from an eventual unwanted level of ZON contamination is suitable for consumption by an animal. The food product can also be a product suspected of comprising an unwanted ZON level, and/or a product having an unknown ZON level, including products not comprising a detectable ZON level. Preferably the food product is a grain based product. Preferably the grain based product comprises cereal(s), e.g., one or more of corn, wheat, barley, rye, rice,

sorghum and millet. Also preferred are grain based products comprising material derived from one or more of corn, wheat, barley, rye, rice, sorghum and millet. In one embodiment, the food product may e.g. be derived solely from cereal(s), and in another embodiment partly from legumes, e.g. from soybean, and partly from cereals. The grain based product may comprise whole or milled grain, e.g., wet or dry milled grain, including grain based product comprising fractions of wet or dry milled grain, e.g., gluten, protein, starch, and/or oil fractions. Also preferred are products comprising a by-product from brewing and/or fermentation processes, e.g., spent grain. Spent grain is the by-products from the production of alcoholic beverages and ethanol fuels. Brewers' spent grain (BSG) is the residue of beer making in breweries, which use malted barley as the major raw material. Distiller's spent grain (DSG) is the product left in distilleries after alcohol is removed by distillation from the fermented grains such as corn, wheat, barley, rice, and rye. Distiller's spent grain is also known as distiller's grain. Wet distiller's grain (WDG) is dried to produce dried distiller's grain (DDG) which is used primarily as animal feed. Under yet another embodiment of the present invention, the feed product is to be used to make processed feed based on starch or meal.

The term “food supplement” as used according to the invention refers to any nutritional or functional supplement to a food product that would improve the uptake or tolerability of the food. Food supplements as used herein typically provide for a functional food product that aids in the prevention and/or treatment of disease conditions associated with mycotoxins. Therefore, the food supplement according to the invention is also called a nutraceutical. In this regard the food supplement according to the invention is used as a nutraceuticals to accomplish animal food and to reduce side effects resulting from an eventual mycotoxin contamination. The food supplement may be commercialized as an additive separate from the food product, with the advantage of the specific dosing to meet the animal’s need on an individual or cohort basis. The food supplement may be admixed to the food product in a liquid, slurry (e.g. with water) or paste, and fed optionally after an incubation time of one to several hours. In a preferred embodiment, an aqueous solution or suspension is kept at a temperature that allows the enzyme to dissolve. The incubation time for *in vitro* processing preferably ranges from 1 minute to 1 week depending on the processing temperature. In many cases a treatment time in the range of 6 to 48 hours will be suitable. Preferably a reaction temperature is applied which is close to the optimum temperature of the enzyme employed. In numerous embodiments of the invention, temperatures in the range of 10 – 65°C, more preferably 25 – 50°C should be employed.

However, the food supplement may also be provided together with the food product as a dry mixture, either in a ready-to-use mixture or as a kit of parts, which provides for dry mixing onsite before feeding. To provide the mixture the food

supplement is typically placed with the grain in a mixing device, such as a rotating drum, an electric mixer or a tumbler.

The ratio of admixing the food additive ranges from 0.01 µg/t to 10 kg/t feed, preferred from 0.01 mg/t to 1000 g/t, more preferred from 0.1 mg/t to 10 g/t, most preferred from 1 mg/t to 1 g/t.

Other ingredients which, though not critical to the function of the enzyme, may be deemed as helpful in assisting in its roles of mycotoxin degradation and management which may be added to the composition include, but are not limited to, varying amounts of plant or algae extracts or diatomaceous earth.

Besides, the animal food additives of the invention preferably contain fat-soluble and/or water soluble vitamins, trace minerals, macro minerals, aroma compounds, stabilizers and co-substrates.

In a preferred embodiment the food supplement is provided in the dry form as a storage stable powder or granules. Preferably the dry enzyme preparation has a water content of less than 5%, more preferred less than 4%, more preferred less than 3%, even more preferred less than 2%.

The term "isolated enzyme" or “purified enzyme” as used herein refers to a polypeptide which is at least 30% pure, preferably at least 50% pure, more preferably at least 70% pure, even more preferably at least 80% pure, most preferably at least 90% pure, and even most preferably at least 95% pure, as determined by SDS-PAGE.

The food product according to the invention typically is provided in the food or feed grade quality. The grade quality is the quality characteristics of food or feed that is acceptable to animals. This includes external factors as appearance (size, shape, color, gloss, and consistency), texture and flavor. Quality standards also provide for an acceptable amount of contaminating substances. Besides ingredient quality, there are also sanitation requirements. It is important to ensure that the food processing environment is as clean as possible in order to produce the safest possible food for the consumer.

The zonase as used according to the invention optionally is of the serine hydrolase superfamily, which is one of the largest known enzyme families. This family particularly includes:

- serine proteases like trypsin,
- lipases like pancreatic lipase, hormone sensitive lipase, and triacylglycerol lipase,
- cutinases,
- esterases,
- acetylcholinesterase,
- thioesterases,
- certain phospholipases like phospholipase A2, and

- some amidases like fatty acid amide hydrolase.

All of these enzymes share a catalytic mechanism that involves a catalytic triad consisting of a serine nucleophile that is activated by a proton relay involving an acidic residue (e.g. aspartate or glutamate) and a basic residue (usually histidine) although variations on this mechanism exist.

The present invention refers to any type of zonases of the serine hydrolase superfamily or functionally active variants thereof, which surprisingly have proven to be GI stable and exert a ZON degrading activity. As used herein the term “enzyme” always includes functionally active variants thereof.

For the purposes of the present invention the ZON degradation activity is determined in the unit of ZDA. One ZDA unit is defined as hydrolysis of 1 µmol ZON per hour at 37°C and pH 7. A suitable ZDA assay is a cell based standard proliferation assay, e.g. as exemplified below. ZDA activity may be determined in food and premix.

The enzyme as used according to the invention typically exerts a ZON degrading activity of at least 40 mU/mg enzyme.

GI stability may be determined by the pH stability and/or pepsin and/ or pancreatic protease stability, e.g. as exemplified below.

The estradiol-like effects of zearalenone are based on the lactone ring structure. ZON is converted to a non-estrogenic product by a detoxifying enzyme, which inactivates the molecule by enzymatic hydrolysis of the ring.

Cleavage of the lactone ring and subsequent decarboxylation could be accomplished by enzymes like lactone esterases or lactone decarboxylases. Some enzymes of the serine hydrolase superfamily active on zearalenone were now identified that were surprisingly GI stable.

In a first step enzyme candidates from various sources were selected, which are able to metabolize and detoxify zearalenone. Therefore a screening assay determining the biological activity of zearalenone as an estradiol-analogue was employed. Commercially available zearalenone (Sigma-Aldrich) was pre-incubated with enzymes of interest, and the mixture was incubated with cell lines susceptible to estrogen treatment (e. g. human breast cancer cell lines). The proliferation of the cells then served as read-out of the assay under high-throughput conditions. Potential hits were further characterized with regards to activity and stability and could be optimized by *in vivo* recombination to produce variants with improved features. These libraries of variants were individually screened for ZON reaction. Alternatively a set of variants may be screened, i.e. libraries of at least 20 zonases. Upon incubating the enzymes with the substrate zearalenone and screening for reaction products by HPLC, the desired detoxification product was verified by mass spectrometry (MS) analysis. The most preferred variants were then biologically characterized.

The esterase BTA-1 hydrolase (Myco 026; SEQ ID NO: 11, 12) of *Thermobifida fusca*, which is known to be a polyesterase, was found to effectively hydrolyze ZON. Other zonase -family members that share high homology in the active center and have some diversity over the whole sequence were identified by homology searches. Thereby, the lipase from *Streptomyces exfoliates* (Myco 24; SEQ ID NO: 7, 8), PBSA depolymerase from *Acidovorax delfieldii* (Myco 025 - SEQ ID NO: 9, 10) and BTA-2 hydrolase from *Thermobifida fusca* (Myco 023; SEQ ID NO: 5, 6) was found to be preferably used according to the invention. Further zonase with at least 50% sequence identity could be preferred, including functionally active variants to hydrolyze the mycotoxin.

Specifically the ZON detoxifying activity was detected for *Thermobifida fusca* BTA-hydrolase 1 (Myco 026; SEQ ID NO: 11, 12), an enzyme of the abH25.1 moxarella lipase 1 like family. Enzymatic cleavage of zearalenone and subsequent decarboxylation lead to a product representing a mass of approximately 290 g/mol. Other family members homologous to BTA-hydrolase 1 were identified by homology searches. It was found that all enzymes share high homology in the active centre, but have enough diversity over the whole sequence to ensure the generation of broad diversity by recombination.

As a preferred example lipase from *Streptomyces exfoliatus* and PBS A depolymerase from *Acidovorax delafieldii* were chosen for homologous recombination in addition to BTA hydrolase from *Thermobifida fusca*.

The lipase of *Streptomyces exfoliatus* lipase exhibits a typical canonical tertiary fold of an α/β hydrolase with a catalytic triad formed by the amino acids Serine 131, Aspartate 177 and Histidine 209. It closely resembles catalytic triads found in other neutral lipases but does not contain a regulatory lid.

The term “variant” or “functionally active variant” of an enzyme as used according to the invention herein means a sequence resulting from modification of the parent sequence by insertion, deletion or substitution of one or more amino acids or nucleotides within the sequence or at either or both of the distal ends of the sequence, and which modification does not affect (in particular impair) the activity of this sequence. In a preferred embodiment the functionally active variant

a) is a biologically active fragment of the amino acid or the nucleotide sequence, the functionally active fragment comprising at least 50% of the sequence of the amino acid or the nucleotide sequence, preferably at least 60%, preferably at least 70%, more preferably at least 80%, still more preferably at least 90%, even more preferably at least 95% and most preferably at least 97%, 98% or 99%;

b) is derived from the amino acid or the nucleotide sequence by at least one amino acid substitution, addition and/or deletion, wherein the functionally active variant has a sequence identity to the amino acid or the nucleotide sequence or to the functionally

active fragment as defined in a) of at least 50%, preferably at least 60%, preferably at least 70%, preferably at least 80%, still more preferably at least 90%, even more preferably at least 95% and most preferably at least 97%, 98% or 99%; and/or
c) consists of the amino acid or the nucleotide sequence and additionally at least one amino acid or nucleotide heterologous to the amino acid or the nucleotide sequence, preferably wherein the functionally active variant is derived from or identical to any of the naturally occurring variants of any of the sequences of SEQ ID NO: 1, 2 and 5 - 12.

"Percent (%) amino acid sequence identity" with respect to the polypeptide sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the specific polypeptide sequence, after aligning the sequence and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

The functionally active variant may be obtained by sequence alterations in the amino acid or the nucleotide sequence, wherein the sequence alterations retains a function of the unaltered amino acid or the nucleotide sequence, when used in combination of the invention. Such sequence alterations can include, but are not limited to (conservative) substitutions, additions, deletions, mutations and insertions.

In a specific embodiment of the invention the polypeptide or the nucleotide sequence as defined above may be modified by a variety of chemical techniques to produce derivatives having essentially the same activity (as defined above for fragments and variants) as the modified polypeptide or the nucleotide sequence, and optionally having other desirable properties. Other desirable properties are, for example, the increase in thermostability and/or GI stability, as measured by the pH stability and/or protease stability of the enzyme.

The variant of the polypeptide or the nucleotide sequence is functionally active in the context of the present invention, if the activity of the composition of the invention including the variant (but not the original) amounts to at least 50%, preferably at least 60%, more preferred at least 70%, still more preferably at least 80%, especially at least 90%, particularly at least 95%, most preferably at least 99% of the activity of the enzyme as used according to the invention including the amino acid or the nucleotide sequence without sequence alteration (i.e. the original polypeptide or the nucleotide sequence).

Functionally active variants may be obtained by changing the sequence as defined above and are characterized by having a biological activity similar to that

displayed by the respective sequence of SEQ ID NO: 1, 2 and 5 - 12 from which the variant is derived, including the ability of ZON hydrolysis in a food product.

In another preferred embodiment of the invention, the functionally active variant of the enzyme as used according to the invention is essentially identical to the amino acid of the SEQ ID NO: 2, 6, 8, 10 or 12, but differs from the amino acid sequence, respectively, in that it is derived from a homologous sequence of a different strain or different species. These are referred to as naturally occurring variants.

Still, the term "functionally active variant" includes naturally occurring allelic variants, as well as mutants or any other non-naturally occurring variants. As is known in the art, an allelic variant is an alternate form of a (poly)peptide that is characterized as having a substitution, deletion, or addition of one or more amino acids that does essentially not alter the biological function of the polypeptide.

In a preferred embodiment, the functionally active variant derived from the amino acid or the nucleotide sequence as defined above by amino acid exchanges, deletions or insertions may also conserve, or more preferably improve, the activity.

Conservative substitutions are those that take place within a family of amino acids that are related in their side chains and chemical properties. Examples of such families are amino acids with basic side chains, with acidic side chains, with non-polar aliphatic side chains, with non-polar aromatic side chains, with uncharged polar side chains, with small side chains, with large side chains, etc.

Among the well-known mutagenesis approaches for evolving new properties in enzymes are *in vivo* homologous recombination, site-directed mutagenesis, random mutagenesis, error prone PCR and mutagenesis approaches based on the use of genetic engineering methods, such as restriction and ligation. According to a preferred embodiment genes are shuffled enabling the molecular mixing of naturally similar or randomly mutated genes, also called mosaic genes. By using such recombination approaches functionally active variants are preferably provided in a library of variants, which can be used to screen for those variants with improved phenotype. Using mutagenesis allows for the generation of new mutated sequences which code for enzymes with altered, preferably improved functions and/or newly acquired functions. In this way it is possible, for example, to achieve improvements in the thermostability of an enzyme, to change the substrate specificity of an enzyme, to improve its activity, to evolve new catalytic sites and/or to fuse domains from two different enzymes.

In accordance therewith, an enzyme sequence is preferably mutagenized to introduce foreign amino acids into the amino acid sequence, e.g. through mutagenesis methods. The term “foreign” in the context of amino acids shall mean the newly introduced amino acids being naturally occurring, but foreign to the site of modification, or substitutes of naturally occurring amino acids. Mutagenesis methods preferably employ homologous recombination, but random, semi-random or, in particular, by site-

directed random mutagenesis methods are also feasible, in particular to delete, exchange or introduce randomly generated inserts into the respective nucleic acid sequences. According to a preferred embodiment sequences of homologous native enzymes are recombined, preferably an N-terminal sequence of one enzyme is combined with a C-terminal sequence of another one with sequence homology, such as a serine hydrolase from another species or another type of serine hydrolases. According to a preferred embodiment the nucleic acid sequences are mutagenized, which are not directly involved in the catalytic center, to improve any characteristics of the enzyme, such as pH or protease stability. However, it is also preferred that sequences of the catalytic center are mutagenized to improve the enzymatic activity.

Particularly preferred are homologous recombination techniques, such as those described in WO03/064667, WO2005/075654}

Therefore homologous DNA sequences are preferred which diverge by at least one nucleotide. In a preferred embodiment of the invention the DNA sequences to be recombined diverge by at least 0.9% up to 35%. Preferably the DNA sequences to be recombined are short sequences with a preferred length in the range of 20 to 3000 nucleotides.

DNA sequences to be recombined may be from natural sources or artificial sequences that are synthetically produced. Natural DNA sequences to be recombined may be derived from any species.

The preferred BTA2 hydrolase variants were produced by homologous recombination with the sequences from the lipase or the PBS A depolymerase. Table 3 provides an overview about preferred recombinant pattern of hits.

A typical library of enzyme variants has a number of enzyme variants of at least 2000 preferably 20000. Using a large library increases the probability of selecting a functionally active variant with improved properties.

Libraries as used according to the invention preferably comprise at least 10^2 library members, more preferred at least 10^3, more preferred at least 10^4, more preferred at least 10^5, more preferred at least 10^6 library members, preferably derived from a parent molecule, which is a functional serine hydrolase as a scaffold to engineer a diverse repertoire of variants which for selecting the best suitable serine hydrolase with improved properties.

The enzyme may be used to detoxify a food product, if there is a risk of ZON contamination. Thus, the food supplement according to the invention is preferably used as a prophylactic measure to avoid unwanted toxic effects, even before determining a ZON concentration in a food product. On the other hand it may be preferred to decontaminate a food product that has a predetermined ZON contamination and could still be used for feeding upon enzymatic treatment.

The *in situ* biotransformation which is effected upon oral administration, i.e. feeding, seems to occur in the small intestine. Therefore, the feed pulp first faces an acid shock in the stomach at a pH 3-5. In the course of digestion in the digestive tract the enzyme activity can then detoxify eventually contaminating mycotoxins before their uptake and metabolism. Animal experiments show that ZON could be degraded immediately upon consumption, i.e. in the stomach.

Ex vivo and animal studies are conducted to evaluate the biological effect of the enzymes as used according to the invention.

The term "recombinant" as used herein refers to enzymes produced by recombination techniques employing host cell expression systems, e.g. in a production host cell line, either having the native sequence or variant sequences produced by recombination.

Therefore, nucleotide sequences corresponding to the enzymes according to the invention are also provided. The nucleotide sequences can be used in expression cassettes for transformation of host cells of interest. In particular, expression cassettes for expression of the sequences in plants and other host organisms are provided as well as transformed plants and other host cells.

The term "host cell" or "hosts cell line" refers to a microorganism, used for expression of a recombinant gene to produce the recombinant enzymes as used according to the invention. Preferred host cells are selected from the group consisting of *Escherichia coli, Bacillus spp., Pichia pastoris*.

A host cell clone of cultivated host cells that have proliferated is commonly understood to be a host cell line. A production host cell line is commonly understood to be a cell line ready-to-use for cultivation in a bioreactor to obtain the enzyme product in an industrial scale.

In one aspect, the present invention features a method to mass produce the recombinant enzyme according to the invention in large scale amounts with appropriate purity to enable large scale production for industrial use. In a broad embodiment, the method comprises the step of transfecting an Open Reading Frame encoding for all or part of such an enzyme into a cell suitable for the expression thereof. In some embodiments, a cDNA encoding for a complete enzyme is used. However, in other embodiments, a cDNA encoding for a biologically active variant thereof may be used. Specifically, one or more amino acid substitutions may be made while preserving or enhancing the biological activity of the enzyme. In other preferred embodiments, an expression vector is used to transfer the cDNA into a suitable cell or cell line for expression thereof. In one particularly preferred embodiment, the cDNA is transfected into E. Coli BL21 to create a production cell line. In yet other preferred embodiments, the production procedure features of pH, continuous perfusion or batch fermentation, macroporous microcarriers may be used to produce cell mass followed

by a shift to a medium for production in a continuous, fed-batch or batch process to produce the recombinant enzyme on a large scale. Accordingly a yield of at least 1 mg per liter of culture per one production run, or more at peak culturing density can be produced starting with a one liter culture system.

Therefore, the present invention also provides for a transfected strain which features the ability to produce the enzyme according to the invention in amounts which enable using the enzyme on an industrial scale. In some preferred embodiments, the strain may contain more than 1 copy of an expression construct. In even more preferred embodiments, the strain expresses the recombinant enzyme in amounts of at least 10 mg/kg biomass.

Expression of the recombinant enzyme according to the invention will also be feasible using yeast, algae, mammalian or plant expression systems, whereby transgenic plants are provided with heterologous genes integrated into the plant genome, such that the resulting plants produce levels of the recombinant enzyme.

In another aspect, the present invention provides novel vectors suitable to produce the enzyme according to the invention in amounts which enable using the enzyme on an industrial scale. In preferred embodiments, the present invention features an expression vector comprising a promoter/enhancer element or other regulatory elements.

The choice of regulatory sequences, such as promoter and terminator sequences and optionally signal or transit sequences are determined, for example, on the basis of when, where, and how the polypeptide is desired to be expressed. For instance, the expression of the gene encoding a polypeptide of the present invention may be constitutive or inducible, or may be developmental, stage or tissue specific, and the gene product may be targeted to a specific cell compartment (intracellular or extracellular), tissue or plant part such as seeds or leaves.

Multiple copy insertions of the vector are preferred to increase the yield.

In another aspect, the present invention provides for the production of a recombinant enzyme according to the invention. The specific activity of the enzyme according to the present invention is at least 10 mU per milligram protein [One unit is defined as hydrolysis of 1 µmol ZON per hour at 37°C and pH 7].

The recombinant enzyme according to the invention preferably is purified by immunoprecipitation, liquid chromatography.

The recombinant enzymes according to the invention are preferably used as a food grade or feed grade material, e.g. obtained upon purification from the host organism.

The foregoing description will be more fully understood with reference to the following examples. Such examples are, however, merely representative of methods of

practicing one or more embodiments of the present invention and should not be read as limiting the scope of invention.

The subject matter of the following definitions is considered embodiments of the present invention:

1. Use of a GI-stable recombinant zonase for treating food products.

2. Use of a zonase according to claim 1, wherein the zonase is selected from the group consisting of hydrolases, lipases, esterases and depolymerases.

3. Use of a zonase according to claim 1 or 2, wherein the zonase is selected from the moraxella lipase 1 like enzyme family consisting of BTA-hydrolase 1 from *Thermobifida fusca*, BTA-hydrolase 2 from *Thermobifida fusca*, lipase from *Streptomyces exfoliatus*, PBS A depolymerase from *Acidovoras delafieldii*, and triacylglycerol hydrolase from *Streptomyces sp.,* or functionally active variants thereof.

4. Use of a zonase according to any of claims 1 to 3, wherein the zonase is stable in the range of pH 3 to 8.

5. A food supplement for treating a potentially mycotoxin contaminated food product comprising an enzyme product containing a zonase according to any of claims 1 to 4.

6. An enzyme product according to claim 5, wherein said enzyme product is provided with the recombinant host, optionally as lysate.

7. The food supplement according to any of claims 1 to 5, wherein the enzyme has a ZON detoxification activity in a cell based assay of at least.

8. A feed product comprising grain and at least one recombinant GI stable zonase.

9. The feed product according to claim 8, which contains the enzyme in a dry mixture.

10. The feed product according to claim 8 or 9, wherein the grain is selected from corn, wheat, barley, rye, rice, sorghum and millet.

11. A functionally active enzyme variant obtainable by *in vivo* recombination of at least two parentals selected from the group consisting of BTA-hydrolase 1 from *Thermobifida fusca*, BTA-hydrolase 2 of *Thermobifida fusca*, lipase from *Streptomyces exfoliatus*, PBS A depolymerase from *Acidovoras delafieldii* and triacylglycerol hydrolase from *Streptomyces sp..*

12. A functionally active enzyme variant according to claim 11, wherein one parental is BTA-hydrolase 2.

13. A functionally active enzyme variant according to claim 12 comprising at least 25 consecutive amino acids selected from the amino acid sequence as set forth in SEQ ID NO: 6 and at least 25 consecutive amino acids selected from the amino acid sequence as set forth in SEQ ID NO: 8 or SEQ ID NO: 10.

14. A functionally active enzyme variant according to claim 13 selected from the group consisting of the amino acid sequence as set forth in SEQ ID NO: 13, 14, 15 and 16.

EXAMPLES

Example 1: Cell based assays to detect the enzymatic detoxification of ZON

For the screening of a repertoire of enzymes, e.g. a recombination library, a fast and sensitive high throughput assay to detect the degradation of zearalenone was developed.

Cloning of pERE-SEAP for estrogen sensitive alkaline phosphatase assay in MCF-7:

The Estrogen receptor enhancer from *Xenopus vitellogenin* A2 (Klinge C.M (2001) Nucleic Acids Res., 29(14):2905-19.) was cloned into the pTAL-SEAP vector from Clonetech.

pTAL-SEAP was designed for analyzing enhancer sequences by assaying for expression of the secreted alkaline phosphatase (SEAP) gene. Estrogens diffuse into the cell, bind to estrogen receptors (ERs) and activate the transcription of the secreted alkaline phosphatase, regulated by estrogen response elements (EREs). The secreted alkaline phosphatase can easily be detected by adding p-nitrophenylphosphate (pNP) substrate to the culture supernatant and measuring the colorimetric change at OD 405 nm in a 96-well plate reader.

To construct the pERE-SEAP plasmid, the pTAL-SEAP vector was cut with NheI and XhoI, purified and ligated with the annealed primers encoding for the estrogen receptor enhancer from Xenopus vitellogenin A2 (5' CTA GCG TCA GGT CAC AGT GAC CTG ATC AAA GTT AAT GTA ACC TCA 3'). MCF-7 cells were stably transfected with the pERE-SEAP plasmid, named MCF7 pERE-SEAP pcDNA #1 cell line.

Final Protocol for the zearalenone degrading MCF-7 ALP Assay:

MCF7 pERE-SEAP pcDNA #1 cells are cultivated in 175 cm^2 flasks (BD) for one week in estrogen free media (RPMI 1640 without phenol red (PAN Biotech GmbH) supplemented with 10% charcoal stripped FCS (PAA), 1% Pen/Strep (PAN Biotech GmbH), 1% 10 mM MEM non-essential amino acids (PAN Biotech GmbH)). 2 µl of 10 pM – 10 µM zearalenone solution (10 mM stock solution in DMSO (Applichem), diluted with estrogen free media) is mixed with 10 µl of zearalenone degrading enzyme preparation in 96-wells at 37°C. After 3 h incubation 90 µl of the MCF7 pERE-SEAP pcDNA #1 cell suspension (0.3 x 10^6 cells/ml estrogen free media) is added to the zearalenone enzyme reaction mix and incubated for 4 days at 37°C.

The alkaline phosphatase secreted into the supernatant of adherent tMCF-7 cells corresponding to the estrogenic activity of zearalenone is measured by the

cleavage of p-nitrophenylphosphate substrate: 50 µl of the culture media is transferred into a 96-well plate (BD) and 50 µl of p-nitrophenylphosphate substrate solution (1 mg pNPP in ALP buffer with 200 mM Glycin, 2 mM $MgCl_2$, 2 mM $ZnCl_2$, pH 9.6 (Sigma)) is added. After incubation for 1 h at 37°C the amount of the yellow p-nitrophenol is measured in a plate reader (Tecan) at OD 405nm.

Example 2: Selection of enzyme candidates for detoxification of zearalenone

Parental enzymes for the recombination were identified which are able to metabolize and detoxify zearalenone. In parallel, high throughput assays were developed for the characterization of the parental enzymes and subsequently the recombinants.

The screening assay of Example 1 makes use of the biological activity of zearalenone as an estradiol-analogue. Commercially available zearalenone (Sigma-Aldrich) is pre-incubated with enzymes of interest, and the mixture is incubated with cell lines susceptible to estrogen treatment (e. g. the MCF7 pERE-SEAP pcDNA #1 cell line). The proliferation of the cells serves as read-out of the assay and helps to identify potential hits. This assay is scaled to high-throughput conditions. Potential hits are further characterized with regards to activity and stability and can be optimized in further rounds of recombination. The leads are then biologically characterized.

Reaction products of ZON were first generated by chemical cleavage of the lactone ring to provide references for the HPLC analysis of enzymatic reactions. After the establishment of the HPLC methods a library of ≥20 different hydrolases was individually incubated with the substrate zearalenone and screened for reaction products by HPLC. The desired detoxification product was ultimately verified by mass spectrometry (MS) analysis.

Zearalenone detoxifying activity was detected for *Thermobifida fusca* BTA-hydrolase 1, an enzyme of the abH25.1 *moxarella* lipase 1 like family.

Other family members homologous to BTA-hydrolase 1 were identified by homology analysis. All enzymes share a high homology in the active centre but have enough diversity over the whole sequence to ensure the generation of a broad diversity by recombination.

Table 1: Identified zearalenone degrading enzymes:

Number	Enzyme	Species
Myco 021	Triacylglycerol acylhydrolase	*Streptomyces sp.*
Myco 022	Zearalenone hydrolase (positive control)	*Bionectria ochroleuca*
Myco 023	BTA-hydrolase 2	*Thermobifida fusca*
Myco 024	Lipase	*Streptomyces exfoliatus*
Myco 025	PBS A depolymerase	*Acidovorax delafieldii*
Myco 026	BTA-hydrolase 1	*Thermobifida fusca*

The active center and the catalytic triad are highly conserved in this family of hydrolases, while a prior art zearalenone hydrolase from *Bionectria ochroleuca* (WO03/080842) co-aligned showed significant differences to the enzyme candidates chosen for recombination.

The identity of the enzyme candidates for zearalenone detoxification is in the range of 42 to 92% at the protein level, which is an appropriate range for an *in vivo* homologous recombination in a mismatch repair deficient recombination system as described in WO03/064667, see Table 2. The lipases from the streptomyces species are the closest relatives, sharing 93% identity, while the PBSA depolymerase from *Acidovorax delafieldii* has the least identical sequence of all four enzyme candidates for recombination (~62-63 % identity).

Table 2: Homology of hydrolases. Identity of each pair of enzymes at protein level is listed. Recombined enzymes are highlighted.

Identity %	**Myco 021**	**Myco 022**	***Myco 023***	***Myco 024***	***Myco 025***	**Myco 026**
Myco 021	100%	16,57	57,46	89,39	43,75	57,14
Myco 022		100%	16,42	16,03	14,08	16,47
Myco 023			100%	**56,59**	**43,75**	92,05
Myco 024				100%	**42,14**	56,59
Myco 025					100%	44,20
Myco 026						100%

All candidate enzymes of Table 1 show ZON degrading activity. Zearalenone hydrolase from *Bionectria ochroleuca* (Myco 022) was used as positive control.

Example 3: Generation of library: Recombination of three enzymes with zearalenone detoxifying activity

To generate the screening library the following three genes were chosen as final candidates for the homologous recombination (see US5965415).

Briefly, in a first step the three genes were cloned into the yeast integration plasmid. After digestion and linearization the genes were transformed into the diploid yeast strain. The transformed yeast cells where then spread on selection plates.

The heterozygous transformants containing the integration of the linearized plasmid DNA at the wanted genomic position were identified by PCR. The yeast strains were cultured and sporulation was induced.

Between 14 and 16 tetrads of each strain were dissected. Haploids carrying all relevant markers, needed for the generation of the wanted heterozygous diploid strains for the recombination assay, were identified on selection plates.

For the recombination the haploid yeast strains were mated and after induction of sporulation the recombination frequency of the different gene combinations was evaluated.

To transform the recombination library into the final expression plasmids for the high throughput screening, the yeast genomic DNA was isolated from large scale spore preparations grown in corresponding selection medium to be used as template for PCR reactions.

In a two-step procedure, amplicons were generated with oligonucleotides annealing to the flanking marker regions first to select for DNA which had crossovers in between the flanking markers. Second, gene-specific oligonucleotides were used to amplify the recombined genes of the enzymes and to outfit the genes with restriction sites for downstream cloning into expression plasmids.

PCR fragments were cloned into pCR4-TOPO (Invitrogen) and transformed into chemically competent DH5a (NEB) cells. The quality of the six libraries was analyzed by DNA sequencing of single colonies. Each of the six libraries consisted of approximately 2000 - 5000 independent clones (table 3).

Table 3: Amount of Independent Clones per Library:

Library	Independent Clones
Myco 023 x Myco 024	> 2000
Myco 023 x Myco 025	> 5000
Myco 024 x Myco 023	> 2000
Myco 024 x Myco 025	> 5000
Myco 025 x Myco 023	> 5000
Myco 025 x Myco 024	> 2000

Example 4: Screening of Recombination Library and Re-Screening of Hits

For the screening of the recombination library the assay of Example 1 is used.

ZON degrading activity assay

To determine the ZON degrading activity MCF7 pERE-SEAP pcDNA #1 cells were cultivated in 175 cm^2 flasks for one week in estrogen free media (RPMI 1640 without phenol red (PAN Biotech GmbH) supplemented with 10% charcoal stripped FCS (PAA), 1% Pen/Strep (PAN Biotech GmbH), 1% 10 mM MEM non-essential amino acids (PAN Biotech GmbH)). 2 µl of 10 pM – 10 µM zearalenone solution (Sigma, 10 mM stock solution in DMSO, diluted with estrogen free media) is mixed

with 10 µl of zearalenone degrading enzyme preparation. After 3 h incubation at 37°C 90 µl of the MCF7 pERE-SEAP pcDNA #1 cell suspension (0.3 x 10^6 cells/ml estrogen free media) was added to the zearalenone enzyme reaction mix and incubated in 96-well plates for 4 days at 37°C.

The alkaline phosphatase secreted into the supernatant of adherent tMCF-7 cells corresponding to the estrogenic activity of zearalenone was measured by the cleave of p-nitrophenylphosphate substrate: 50 µl of the culture media was transferred into a 96-well plate and 50 µl of p-nitrophenylphosphate substrate solution (1 mg pNPP in 2xALP buffer with 200 mM Glycerol, 2 mM $MgCl_2$, 2 mM $ZnCl_2$, pH 9.6; Sigma) was added. After incubation for 1 h at 37°C the amount of the yellow p-nitrophenol is measured in a plate reader (Tecan) at OD 405nm.

p-NP-butyrate Assay:

The p-nitrophenyl-butyrate assay is a fast and convenient assay to measure the expression of the zearalenone hydrolyzing enzymes: 90 µl 0.5 mM pNP-butyrate substrate (Sigma) in 20 mM Tris buffer with 300 mM NaCl pH7 was added to 10 µl of enzyme solution, e.g. cleared lysate. After incubation for 20 min at 37°C the amount of the hydrolyzed p-nitrophenyl-butyrate ester, the yellow p-nitrophenol was measured in a plate reader (Tecan) at OD 405nm.

Expression of zonases

The following expression systems were used to express the zonases:

Zonases were expressed intracellularly in either pBAD-HisB (Invitrogen) or pTrcHis2A (Invitrogen). For expression in pBAD-HisB (Invitrogen) the inserts were cut by BamHI and HindIII (NEB) for ligation with the vector cut by BglII and HindIII (NEB) and transformed into *E. coli* Top10 as recommended by Invitrogen. To express the enzymes in pTrcHis2A (Invitrogen), the genes were cloned with and without the signal peptide sequence. pTrcHis2A cut with BamHI and SalI was ligated with the genes which were cut by BamHI and XhoI.

All six libraries were cloned into pBad-His expression-vector. To estimate the number of clones expressing active recombinant enzymes 86 clones of each of the recombination libraries Myco 023 x Myco 024, Myco 024 x Myco 023, Myco 023 x Myco 025, Myco 025 x Myco 023 expressed by pBAD/Top 10 have been tested for hydrolysing activity with pNP-butyrate:

86 clones of each library, 10 wells for controls (TB/Amp only, pBad empty vector, parental enzymes Myco 023, Myco 024 and Myco 022 as positive control) were cultivated in 150 µl TB/Amp overnight, diluted 1:20 into 1 ml TB/Amp in a deep well plate, incubated for 2.5 h at 37°C, induced with 0.2% arabinose and further cultivated for 18 h at 25°C 250 rpm. To lyse the pellet 2 mg/ml lysozyme and a 12-finger sonotrode was used. The lysed cells were centrifuged and cleared lysate was tested in

pNP-butyrate assay. As shown in table 4 the number of active clones was dependent on the direction of the parental enzymes.

Table 4: Percentage of active clones in pNP-butyrate assay:

pBAD/Top10	Myco 023 x Myco 024	Myco 024 x Myco 023	Myco 023 x Myco 025	Myco 025 x Myco 023
	62%	85%	1%	25%

High Throughput Screening

For the high throughput screening of the libraries the enzymes were expressed in 96-deep well plates containing 1 ml culture medium.

Freshly transformed recombination library (comprising 6048 clones plus controls) was grown on LB/Amp agar plates overnight to pick single colonies into 96-well plates with 150 µl TB/Amp/well and incubated at 37°C 220 rpm overnight (controls on each plate: TB/Amp only, pBad empty vector, parental enzymes Myco 023 and Myco 024, Myco 022 as positive control).

The overnight culture was then diluted 1:20 into 1 ml TB/Amp in a 96-deep well plate, and directly induced with 0.2% arabinose, incubated for 2 h at 37°C and further cultivated for 18 h at 25°C 220 rpm. The rest of the overnight culture was supplemented with glycerol and the clones were stored at -80°C.

The cell pellet was lysed after a centrifugation step by adding 200 µl lysis buffer (20 mM Tris, 300 mM NaCl, pH7, with 2 mg/ml lysozyme at 4°C) into the deep wells, resuspended and transferred into a V-shape 96-well plate. A 12-finger sonotrode was used to homogenize the lysate and centrifuged. The cleared lysate was transferred to a new 96-well plate and tested with pNP-butyrate assay for activity at pH 7, stability at pH 3 for 30 min at 37°C and thermostability at 70°C for 30 min.

In a second step the cleared lysate was centrifuged through a 96-well sterile filter plate and 10 µl of the filtrate was added to 2 µl zearalenone dilution. After 3 h incubation 90 µl of the MCF7 pERE-SEAP pcDNA #1 cell suspension (0.3×10^6 cells/ml estrogen free media) was added to the zearalenone enzyme reaction mix and incubated in 96-well plates at 37°C.

After 4 days of incubation 50 µl of the culture media was transferred into a 96-well plate and 50 µl of p-nitrophenylphosphate substrate solution (1 mg pNP in 2xALP buffer with 200 mM glycerol, 2 mM $MgCl_2$, 2 mM $ZnCl_2$, pH 9.6) was added. After incubation for 1 h at 37°C the amount of p-nitrophenol was measured in a plate reader (Tecan) at OD 405nm.

Of the 6048 clones tested in the first screen, 1344 clones were selected for rescreening with the zearalenone/ALP assay. pNP-butyrate assay for activity at pH 7, stability at pH 3 for 30 min at 37°C and thermostability at 70°C for 30 min was repeated. Finally 4 Hits were selected for detailed characterization.

Purification of hits

Four hits (Myco H03, Myco H04, Myco H10, Myco H11) and the controls (empty vector, parental enzymes Myco 023, Myco 024 and Myco 022 as positive control) were expressed in pTrc His2a in 1 L Terrific Broth media at 37°C 170 rpm in 5 L shake flasks with buffles. After 3 h an OD600 of approximately 2.5 was reached, the temperature was reduced to 25°C and expression was induced by 1 mM IPTG (final concentration). After 17 h the cells were harvested by centrifugation.

The cell pellet (7 - 9 g wet weight) was resuspended in 50 mM phosphate buffer with 300 mM NaCl, 2mg/ml lysozyme, 20µg/ml DNase and protease inhibitor and treated in a microfluidizer to break the cells (3 passages at 1300 bar). The cell lysate was cleared by centrifugation and the enzyme was purified using the Co^{2+} Talon superflow metal affinity matrix (Clonetech).

The cleared lysate was incubated with the matrix overnight at 4°C, washed twice with buffer without imidazole and the bound enzyme was eluted with 150 mM imidazole in 50 mM phosphate buffer with 300 mM NaCl. The buffer was exchanged by gel filtration on PD-10 columns to 20 mM Tris buffer with 300 mM NaCl, pH7, which was more suitable for the MCF-7 cells.

Purification was confirmed by SDS-PAGE, western blot analysis, amido black staining, protein determination and pNP-butyrate assay.

Thermostability

To measure the thermostability 10 µl purified enzyme (0.5 mg/ml) was mixed with/without 10 µl cleared lysate of *E. coli* with empty plasmid and incubated at 50°C, for 5, 15 and 30 minutes. After a centrifugation step at 4000g to remove aggregates, 70 µl 20mM Tris/HCl 300 mM NaCl, pH 7.0 was added and the reaction was started with 10 µl 5 mM pNP-butyrate. Activity was measured at OD 405nm (37°C) for up to 20 min in a plate reader (Tecan).

As shown in table 5, two of the clones, namely Myco H03 and Myco H11, demonstrated higher thermostability than reference and parental enzymes retaining full activity after 30 min at 50°C.

Table 5: Activity after incubation of purified enzymes at 50°C relative to activity after incubation at 4°C.

	Relative Activity [%] at 50°C		
	5 min	15 min	30 min
Myco 022	33	35	28
Myco 023	100	92	92
Myco 024	16	12	11
Myco H03	98	100	100
Myco H11	86	100	100

pH Stability

pH stability was measured by mixing 10 µl of purified enzyme (0.5 mg/ml) with 40 µl buffer (pH 3, 4, 5, 6, 7 and 8) and incubated for 30 min at 37°C. The pH was then adjusted to pH 8 by addition of 40 µl 1M Tris buffer. The reaction was started with 10 µl 5 mM pNP-butyrate and activity was measured at OD 405nm (37°C) for up to 20 min in a plate reader (Tecan).

As shown in table 6 the enzymes were stable over a broad pH range, with the hits retaining up to 75% residual activity at pH 3 and pH 4. In contrast, the reference enzyme was rapidly inactive at pH 3-5.

Table 6: Residual activity after incubation for 30 min at specified pH.

	Relative Activity [%]					
	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8
Myco 022	0	17	0	89	100	56
Myco 023	49	48	85	99	100	96
Myco 024	42	21	100	129	100	103
Myco H03	64	61	91	96	100	100
Myco H10	64	57	96	99	100	98
Myco H11	75	73	91	93	100	100

Protease Stability

To test protease stability 12.5 µl purified enzyme (0.5 mg/ml) was mixed with or without 12.5 µl cleared lysate of *E. coli* (empty plasmid) and 25 µl pepsin solution (1 mg/ml, 0.1M HCl, pH 2, Sigma) or pancreatin solution (5 mg/ml 0.1 M $NaHCO_3$, pH 8.0, Sigma) was added to the enzyme solution and incubated at 37°C. Aggregates were removed by centrifugation at 4000 g and pH was shifted to pH 8 by adding 40 µl 1 M Tris/HCl 300 mM NaCl, pH 8.0. The reaction was started with 10 µl 5 mM pNP-butyrate and activity was measured at OD 405nm (37°C) for up to 20 min in a plate reader (Tecan).

As shown in table 7 and 8, the parental enzymes and all recombinants were very stable in the presence of pepsin (5 – 30 min) and pancreatin (30 – 120 min) in the presence of cleared lysate from *E. coli*. All enzymes were more stable than the reference enzyme Myco 022.

Table 7: Activity of purified enzymes in cleared lysate of BL21 after incubation with pepsin for 0-30 min.

	Relative Activity [%]			
	0 min	5 min	15 min	30 min
Myco 022	100	100	0	0
Myco 023	100	100	100	100
Myco 024	100	100	100	100
Myco H03	100	100	100	100
Myco H04	100	100	100	100
Myco H10	100	100	100	84
Myco H11	100	100	100	98

Table 8: Activity of purified enzymes in cleared lysate of BL21 after incubation with pancreatin for 0 - 120 min.

	Relative Activity [%]			
	0 min	30 min	60 min	120 min
Myco 022	100	100	41	68
Myco 023	100	27	36	11
Myco 024	100	100	90	100
Myco H03	100	100	100	100
Myco H04	100	93	100	100
Myco H11	100	100	100	100

Claims:

1. Use of a GI-stable recombinant zonase for treating food products.

2. Use of a zonase according to claim 1, wherein the zonase is selected from the group consisting of hydrolases, lipases, esterases and depolymerases.

3. Use of a zonase according to claim 1 or 2, wherein the zonase is selected from the moraxella lipase 1 like enzyme family consisting of BTA-hydrolase 1 from *Thermobifida fusca*, BTA-hydrolase 2 from *Thermobifida fusca*, lipase from *Streptomyces exfoliatus*, PBS A depolymerase from *Acidovoras delafieldii*, and triacylglycerol hydrolase from *Streptomyces sp.,* or functionally active variants thereof.

4. Use of a zonase according to any of claims 1 to 3, wherein the zonase is stable in the range of pH 3 to 8.

5. A food supplement for treating a potentially mycotoxin contaminated food product comprising an enzyme product containing a zonase according to any of claims 1 to 4.

6. An enzyme product according to claim 5, wherein said enzyme product is provided with the recombinant host, optionally as lysate.

7. The food supplement according to any of claims 1 to 5, wherein the enzyme has a ZON detoxification activity in a cell based assay of at least.

8. A feed product comprising grain and at least one recombinant GI stable zonase.

9. The feed product according to claim 8, which contains the enzyme in a dry mixture.

10. The feed product according to claim 8 or 9, wherein the grain is selected from corn, wheat, barley, rye, rice, sorghum and millet.

11. A functionally active enzyme variant obtainable by *in vivo* recombination of at least two parentals selected from the group consisting of BTA-hydrolase 1 from *Thermobifida fusca*, BTA-hydrolase 2 of *Thermobifida fusca*, lipase from *Streptomyces exfoliatus*, PBS A depolymerase from *Acidovoras delafieldii* and triacylglycerol hydrolase from *Streptomyces sp..*

12. A functionally active enzyme variant according to claim 11, wherein one parental is BTA-hydrolase 2.

13. A functionally active enzyme variant according to claim 12 comprising at least 25 consecutive amino acids selected from the amino acid sequence as set forth in SEQ ID NO: 6 and at least 25 consecutive amino acids selected from the amino acid sequence as set forth in SEQ ID NO: 8 or SEQ ID NO: 10.

14. A functionally active enzyme variant according to claim 13 selected from the group consisting of the amino acid sequence as set forth in SEQ ID NO: 13, 14, 15 and 16.

FIGURES:

Fig. 1: Detoxification of zearalenone by cleavage of the lactone ring and decarboxylation.

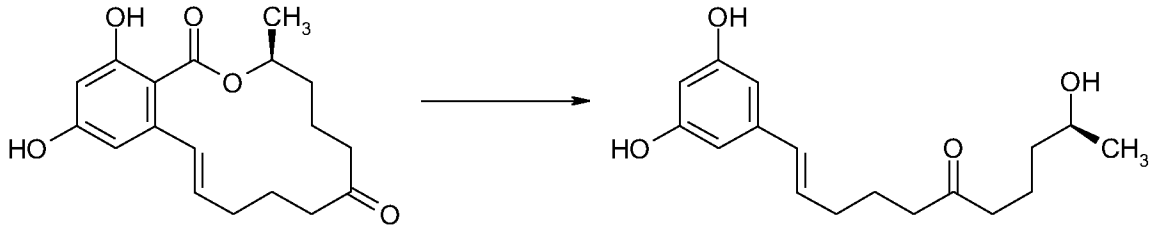

Fig. 2:

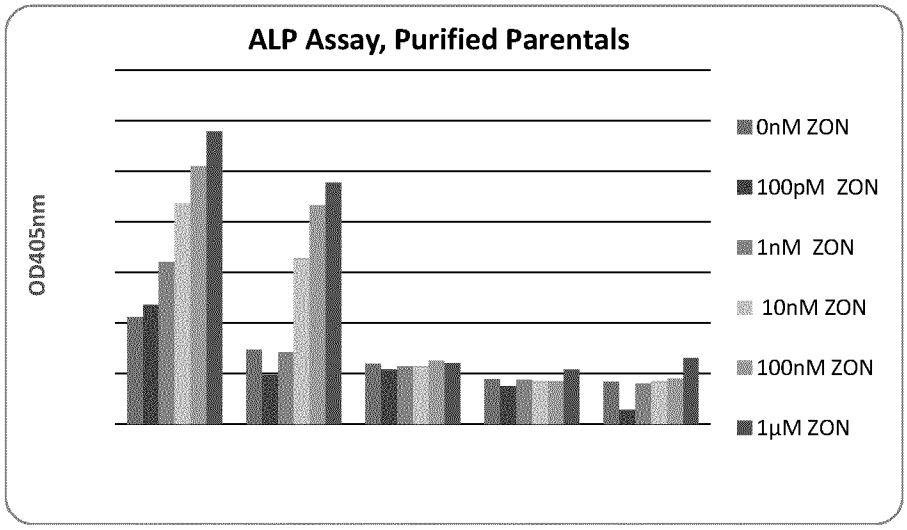

Fig. 3:

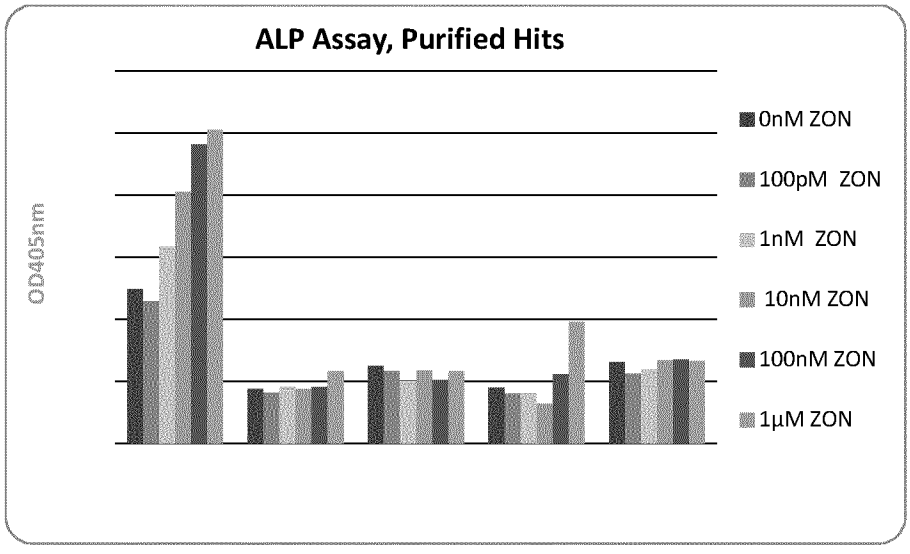

Fig. 4: Sequence alignment parentals and hits

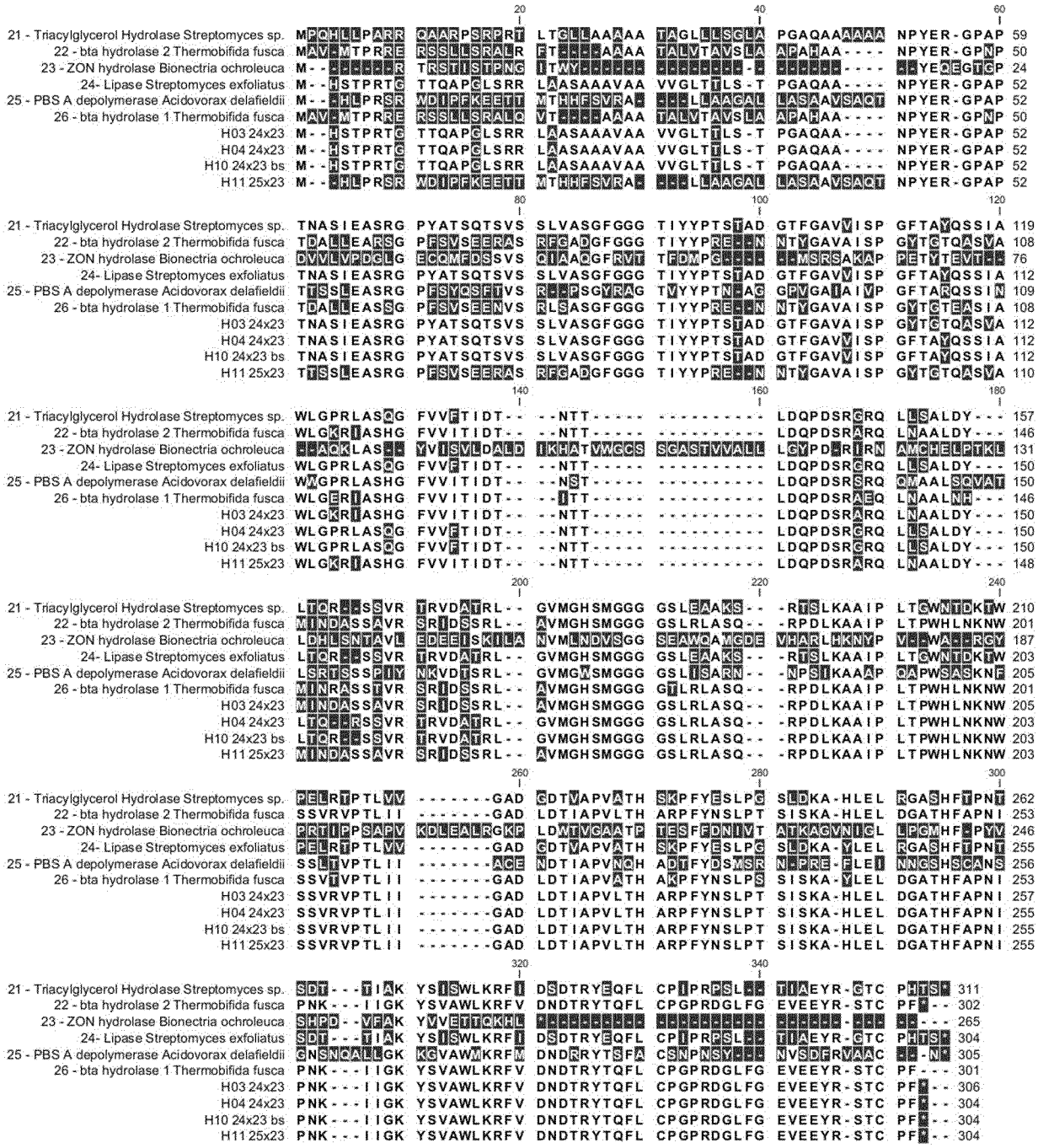

Fig. 5:

SEQ ID NO: 1 - Myco 021

ATGCCGCAGCACCTGCTGCCGGCTCGTCGTCAGGCTGCTCGTCCGTCTCGTCCG
CGTACCCTGACCGGTCTGCTGGCTGCTGCTGCTGCTACCGCTGGTCTGCTGCTG
TCTGGTCTGGCTCCGGGTGCTCAGGCTGCTGCTGCTGCTGCTAACCCGTACGAA
CGTGGTCCGGCTCCGACCAACGCTTCTATCGAGGCTTCTCGTGGTCCGTACGCT
ACCTCTCAGACCTCTGTTTCTTCTCTGGTTGCTTCTGGTTTCGGTGGTGGTACCAT
CTACTACCCGACCTCTACCGCTGACGGTACCTTCGGTGCTGTTGTTATCTCTCCG
GGTTTCACCGCTTACCAGTCTTCTATCGCTTGGCTGGGTCCGCGTCTGGCTTCTC
AGGGTTTCGTTGTTTTCACCATCGACACCAACACCACCCTGGACCAGCCGGACTC
TCGTGGTCGTCAGCTGCTGTCTGCTCTGGACTACCTGACCCAGCGTTCTTCTGTT
CGTACCCGTGTTGACGCTACCCGTCTGGGTGTTATGGGTCACTCTATGGGTGGT
GGTGGTTCTCTGGAAGCTGCTAAATCTCGTACCTCTCTGAAAGCTGCTATCCCGC
TGACCGGTTGGAACACCGACAAAACCTGGCCGGAACTGCGTACCCCGACCCTGG
TTGTTGGTGCTGACGGTGACACCGTTGCTCCGGTTGCTACCCACTCTAAACCGTT
CTACGAATCTCTGCCGGGTTCTCTGGACAAAGCTCACCTGGAACTGCGTGGTGCT
TCTCACTTCACCCCGAACACCTCTGACACCACCATCGCTAAATACTCTATCTCTTG
GCTGAAACGTTTCATCGACTCTGACACCCGTTACGAACAGTTCCTGTGCCCGATC
CCGCGTCCGTCTCTGACCATCGCTGAATACCGTGGTACCTGCCCGCACACCTCTT
GA

Fig. 6:

SEQ ID NO: 2 - Myco 021

MPQHLLPARRQAARPSRPRTLTGLLAAAAATAGLLLSGLAPGAQAAAAAANPYERGP
APTNASIEASRGPYATSQTSVSSLVASGFGGGTIYYPTSTADGTFGAVVISPGFTAYQ
SSIAWLGPRLASQGFVVFTIDTNTTLDQPDSRGRQLLSALDYLTQRSSVRTRVDATRL
GVMGHSMGGGGSLEAAKSRTSLKAAIPLTGWNTDKTWPELRTPTLVVGADGDTVAP
VATHSKPFYESLPGSLDKAHLELRGASHFTPNTSDTTIAKYSISWLKRFIDSDTRYEQF
LCPIPRPSLTIAEYRGTCPHTS*

Fig. 7:

SEQ ID NO: 3 - Myco 022

ATGCGCACTCGCAGCACAATCTCGACCCCGAATGGCATCACCTGGTACTATGAGC
AGGAGGGTACTGGACCCGACGTTGTCCTCGTTCCCGATGGCCTCGGAGAATGCC

AGATGTTTGACAGCTCCGTGTCGCAGATTGCTGCTCAAGGCTTCAGGGTCACTAC
GTTTGATATGCCCGGAATGTCCCGGTCTGCCAAGGCACCACCCGAGACCTACAC
TGAGGTCACGGCCCAGAAGCTGGCTTCCTATGTCATCTCCGTCCTGGATGCTCTT
GACATCAAGCACGCTACTGTCTGGGGCTGCAGCTCAGGAGCTTCCACCGTCGTG
GCGCTGTTGCTCGGTTACCCCGACAGGATACGCAACGCCATGTGCCACGAACTG
CCAACAAAGCTACTGGACCACCTTTCAAACACCGCTGTGCTCGAAGACGAGGAAA
TCTCAAAGATCCTGGCCAATGTAATGTTGAACGACGTGTCTGGAGGCTCGGAGGC
GTGGCAAGCCATGGGGGACGAGGTGCACGCGAGACTGCACAAGAACTACCCGG
TTTGGGCTCGAGGATACCCTCGGACTATTCCTCCCTCAGCTCCGGTTAAGGATCT
GGAGGCTCTGCGTGGGAAGCCCCTGGACTGGACTGTCGGCGCTGCGACACCAA
CCGAGTCTTTCTTTGACAACATTGTTACCGCTACCAAGGCTGGTGTCAACATTGG
GTTGCTTCCAGGGATGCATTTCCCTTATGTTTCCCACCCGGACGTTTTCGCTAAAT
ATGTTGTGGAAACTACGCAGAAGCATCTTTGA

Fig. 8:

SEQ ID NO4: - Myco 022

MRTRSTISTPNGITWYYEQEGTGPDVVLVPDGLGECQMFDSSVSQIAAQGFRVTTFD
MPGMSRSAKAPPETYTEVTAQKLASYVISVLDALDIKHATVWGCSSGASTVVALLLGY
PDRIRNAMCHELPTKLLDHLSNTAVLEDEEISKILANVMLNDVSGGSEAWQAMGDEV
HARLHKNYPVWARGYPRTIPPSAPVKDLEALRGKPLDWTVGAATPTESFFDNIVTAT
KAGVNIGLLPGMHFPYVSHPDVFAKYVVETTQKHL*

Fig. 9:

SEQ ID NO5: - Myco 023

ATGGCTGTTATGACCCCGCGTCGTGAACGTTCTTCTCTGCTGTCTCGTGCTCTGC
GTTTCACCGCTGCTGCTGCTACCGCTCTGGTTACCGCTGTTTCTCTGGCTGCTCC
GGCTCACGCTGCTAACCCGTACGAACGTGGTCCGAACCCGACCGACGCTCTGCT
GGAAGCTCGTTCTGGTCCGTTCTCTGTTTCTGAAGAACGTGCTTCTCGTTTCGGT
GCTGACGGTTTCGGTGGTGGTACCATCTACTACCCGCGTGAAAACAACACCTACG
GTGCTGTTGCTATCTCTCCGGGTTACACCGGTACCCAGGCTTCTGTTGCTTGGCT
GGGTAAACGTATCGCTTCTCACGGTTTCGTTGTTATCACCATCGACACCAACACC
ACCCTGGACCAGCCGGACTCTCGTGCTCGTCAGCTGAACGCTGCTCTGGACTAC
ATGATCAACGACGCTTCTTCTGCTGTTCGTTCTCGTATCGACTCTTCTCGTCTGGC
TGTTATGGGTCACTCTATGGGTGGTGGTGGTTCTCTGCGTCTGGCTTCTCAGCGT

CCGGACCTGAAAGCTGCTATCCCGCTGACCCCGTGGCACCTGAACAAAAACTGG
TCTTCTGTTCGTGTTCCGACCCTGATCATCGGTGCTGACCTGGACACCATCGCTC
CGGTTCTGACCCACGCTCGTCCGTTCTACAACTCTCTGCCGACCTCTATCTCTAA
AGCTCACCTGGAACTGGACGGTGCTACCCACTTCGCTCCGAACATCCCGAACAAA
ATCATCGGTAAATACTCTGTTGCTTGGCTGAAACGTTTCGTTGACAACGACACCC
GTTACACCCAGTTCCTGTGCCCAGGTCCGCGTGACGGTCTGTTCGGTGAAGTTG
AAGAATACCGTTCTACCTGCCCGTTCTGA

Fig. 10:

SEQ ID NO6: - Myco 023

MAVMTPRRERSSLLSRALRFTAAAATALVTAVSLAAPAHAANPYERGPNPTDALLEA
RSGPFSVSEERASRFGADGFGGGTIYYPRENNTYGAVAISPGYTGTQASVAWLGKRI
ASHGFVVITIDTNTTLDQPDSRARQLNAALDYMINDASSAVRSRIDSSRLAVMGHSMG
GGGSLRLASQRPDLKAAIPLTPWHLNKNWSSVRVPTLIIGADLDTIAPVLTHARPFYNS
LPTSISKAHLELDGATHFAPNIPNKIIGKYSVAWLKRFVDNDTRYTQFLCPGPRDGLFG
EVEEYRSTCPF*

Fig. 11:

SEQ ID NO7: - Myco 024

ATGCACTCTACCCCGCGTACCGGTACCACCCAGGCTCCGGGTCTGTCTCGTCGT
CTGGCTGCTTCTGCTGCTGCTGTTGCTGCTGTTGTTGGTCTGACCACCCTGTCTA
CCCCTGGTGCTCAGGCTGCTAACCCGTACGAACGTGGTCCGGCTCCGACCAACG
CTTCTATCGAGGCTTCTCGTGGTCCGTACGCTACCTCTCAGACCTCTGTTTCTTCT
CTGGTTGCTTCTGGTTTCGGTGGTGGTACCATCTACTACCCGACCTCTACCGCTG
ACGGTACCTTCGGTGCTGTTGTTATCTCTCCGGGTTTCACCGCTTACCAGTCTTCT
ATCGCTTGGCTGGGTCCGCGTCTGGCTTCTCAGGGTTTCGTTGTTTTCACCATCG
ACACCAACACCACCCTGGACCAGCCGGACTCTCGTGGTCGTCAGCTGCTGTCTG
CTCTGGACTACCTGACCCAGCGTTCTTCTGTTCGTACCCGTGTTGACGCTACCCG
TCTGGGTGTTATGGGTCACTCTATGGGTGGTGGTGGTTCTCTGGAAGCTGCTAAA
TCTCGTACCTCTCTGAAAGCTGCTATCCCGCTGACCGGTTGGAACACCGACAAAA
CCTGGCCGGAACTGCGTACCCCGACCCTGGTTGTTGGTGCTGACGGTGACACCG
TTGCTCCGGTTGCTACCCACTCTAAACCGTTCTACGAATCTCTGCCGGGTTCTCT
GGACAAGGCTTACCTGGAACTGCGTGGTGCTTCTCACTTCACCCCGAACACCTCT
GACACCACCATCGCTAAATACTCTATCTCTTGGCTGAAACGTTTCATCGACTCTGA

CACCCGTTACGAACAGTTCCTGTGCCCGATCCCGCGTCCGTCTCTGACCATCGCT
GAATACCGTGGTACCTGCCCGCACACCTCTTGA

Fig. 12:

SEQ ID NO8: - Myco 024

MHSTPRTGTTQAPGLSRRLAASAAAVAAVVGLTTLSTPGAQAANPYERGPAPTNASI
EASRGPYATSQTSVSSLVASGFGGGTIYYPTSTADGTFGAVVISPGFTAYQSSIAWLG
PRLASQGFVVFTIDTNTTLDQPDSRGRQLLSALDYLTQRSSVRTRVDATRLGVMGHS
MGGGGSLEAAKSRTSLKAAIPLTGWNTDKTWPELRTPTLVVGADGDTVAPVATHSK
PFYESLPGSLDKAYLELRGASHFTPNTSDTTIAKYSISWLKRFIDSDTRYEQFLCPIPR
PSLTIAEYRGTCPHTS*

Fig. 13:

SEQ ID NO9: - Myco 025

ATGCACCTGCCGCGTTCTCGTTGGGACATCCCGTTCAAAGAAGAAACCACCATGA
CCCACCACTTCTCTGTTCGTGCTCTGCTGGCTGCTGGTGCTCTGCTGGCTTCTGC
TGCTGTTTCTGCTCAGACCAACCCGTACGAACGTGGTCCGGCTCCGACCACCTCT
TCTCTGGAAGCATCTCGTGGTCCGTTCTCTTACCAGTCTTTCACCGTTTCTCGTCC
GTCTGGTTACCGTGCTGGTACCGTTTACTACCCGACCAACGCTGGTGGTCCGGTT
GGTGCTATCGCTATCGTTCCGGGTTTCACCGCTCGTCAGTCTTCTATCAACTGGT
GGGGTCCGCGTCTGGCTTCTCACGGTTTCGTTGTTATCACCATCGACACCAACTC
TACCCTGGACCAGCCGGACTCTCGTTCTCGTCAGCAGATGGCTGCTCTGTCTCAG
GTTGCTACCCTGTCTCGTACCTCTTCTTCTCCGATCTACAACAAAGTTGACACCTC
TCGTCTGGGTGTTATGGGTTGGTCTATGGGTGGTGGTGGTTCTCTGATCTCTGCT
CGTAACAACCCGTCTATCAAAGCTGCTGCTCCGCAGGCTCCGTGGTCTGCTTCTA
AAAACTTCTCTTCTCTGACCGTTCCGACCCTGATCATCGCTTGCGAAAACGACAC
CATCGCTCCGGTTAACCAGCACGCTGACACCTTCTACGACTCTATGTCTCGTAAC
CCGCGTGAGTTCCTGGAAATCAACAACGGTTCTCACTCTTGCGCTAACTCTGGTA
ACTCTAACCAGGCTCTGCTGGGTAAAAAAGGTGTTGCTTGGATGAAACGTTTCAT
GGACAACGACCGTCGTTACACCTCTTTCGCTTGCTCTAACCCGAACTCTTACAAC
GTTTCTGACTTCCGTGTTGCTGCTTGCAACTGA

Fig. 14:

SEQ ID NO10: - Myco 025

MHLPRSRWDIPFKEETTMTHHFSVRALLAAGALLASAAVSAQTNPYERGPAPTTSSL
EASRGPFSYQSFTVSRPSGYRAGTVYYPTNAGGPVGAIAIVPGFTARQSSINWWGP
RLASHGFVVITIDTNSTLDQPDSRSRQQMAALSQVATLSRTSSSPIYNKVDTSRLGVM
GWSMGGGGSLISARNNPSIKAAAPQAPWSASKNFSSLTVPTLIIACENDTIAPVNQHA
DTFYDSMSRNPREFLEINNGSHSCANSGNSNQALLGKKGVAWMKRFMDNDRRYTS
FACSNPNSYNVSDFRVAACN*

Fig. 15:

SEQ ID NO11: - Myco 026

ATGGCTGTGATGACCCCCCGCCGGGAGCGCTCTTCCCTGCTCTCCCGAGCTCTG
CAAGTGACGGCTGCGGCTGCCACAGCGCTTGTGACCGCGGTCAGCCTGGCCGC
CCCCGCTCATGCCGCCAACCCCTACGAGCGCGGCCCCAACCCGACCGACGCCC
TGCTCGAAGCCAGCAGCGGCCCCTTCTCCGTCAGCGAGGAGAACGTCTCCCGGT
TGAGCGCCAGCGGCTTCGGCGGCGGCACCATCTACTACCCGCGGGAGAACAAC
ACCTACGGTGCGGTGGCGATCTCCCCCGGCTACACCGGCACTGAGGCTTCCATC
GCCTGGCTGGGCGAGCGCATCGCCTCCCACGGCTTCGTCGTCATCACCATCGAC
ACCATCACCACCCTCGACCAGCCGGACAGCCGGGCAGAGCAGCTCAACGCCGC
GCTGAACCACATGATCAACCGGGCGTCCTCCACGGTGCGCAGCCGGATCGACAG
CAGCCGACTGGCGGTCATGGGCCACTCCATGGGCGGCGGCGGCACCCTGCGTC
TGGCCTCCCAGCGTCCCGACCTGAAGGCCGCCATCCCGCTCACCCCGTGGCAC
CTCAACAAGAACTGGAGCAGCGTCACCGTGCCGACGCTGATCATCGGGGCCGAC
CTCGACACAATCGCGCCGGTCGCCACGCACGCGAAACCGTTCTACAACAGCCTG
CCGAGCTCCATCAGCAAGGCCTACCTGGAGCTGGACGGCGCAACCCACTTCGCC
CCGAACATCCCCAACAAGATCATCGGCAAGTACAGCGTCGCCTGGCTCAAGCGG
TTCGTCGACAACGACACCCGCTACACCCAGTTCCTCTGCCCCGGACCGCGCGAC
GGACTCTTCGGCGAGGTCGAAGAGTACCGCTCCACCTGCCCGTTCTAG

Fig. 16:

SEQ ID NO12: - Myco 026

MAVMTPRRERSSLLSRALQVTAAAATALVTAVSLAAPAHAANPYERGPNPTDALLEA
SSGPFSVSEENVSRLSASGFGGGTIYYPRENNTYGAVAISPGYTGTEASIAWLGERIA
SHGFVVITIDTITTLDQPDSRAEQLNAALNHMINRASSTVRSRIDSSRLAVMGHSMGG
GGTLRLASQRPDLKAAIPLTPWHLNKNWSSVTVPTLIIGADLDTIAPVATHAKPFYNSL

PSSISKAYLELDGATHFAPNIPNKIIGKYSVAWLKRFVDNDTRYTQFLCPGPRDGLFGE
VEEYRSTCPF

Fig. 17:

SEQ ID NO: 13 - Myco H03

AANPYERGPAPTNASIEASRGPYATSQTSVSSLVASGFGGGTIYYPTSTADGTFGAV
AISPGYTGTQASVAWLGKRIASHGFVVITIDTNTTLDQPDSRARQLNAALDYMINDASS
AVRSRIDSSRLAVMGHSMGGGGSLRLASQRPDLKAAIPLTPWHLNKNWSSVRVPTLII
GADLDTIAPVLTHARPFYNSLPTSISKAHLELDGATHFAPNIPNKIIGKYSVAWLKRFVD
NDTRYTQFLCPGPRDGLFGEVEEYRSTCPF

Fig. 18:

SEQ ID NO: 14 - Myco H04

AANPYERGPAPTNASIEASRGPYATSQTSVSSLVASGFGGGTIYYPTSTADGTFGAV
VISPGFTAYQSSIAWLGPRLASQGFVVFTIDTNTTLDQPDSRGRQLLSALDYLTQRSS
VRTRVDATRLGVMGHSMGGGGSLRLASQRPDLKAAIPLTPWHLNKNWSSVRVPTLII
GADLDTIAPVLTHARPFYNSLPTSISKAHLELDGATHFAPNIPNKIIGKYSVAWLKRFVD
NDTRYTQFLCPGPRDGLFGEVEEYRSTCPF

Fig. 19:

SEQ ID NO: 15 - Myco H10

AANPYERGPAPTNASIEASRGPYATSQTSVSSLVASGFGGGTIYYPTSTADGTFGAV
VISPGFTAYQSSIAWLGPRLASQGFVVFTIDTNTTLDQPDSRGRQLLSALDYLTQRSS
VRTRVDATRLGVMGHSMGGGGSLEAAKSRTSLKAAIPLTGWNTDKTWPELRTPTLV
VGADGDTVAPVATHSKPFYESLPGSLDKAYLELRGASHFTPNTSDTTIAKYSISWLKR
FIDSDTRYEQFLCPGPRDGLFGEVEEYRSTCPF

Fig. 20:

SEQ ID NO: 16 - Myco H11

AQTNPYERGPAPTTSSLEASRGPFSVSEERTSRFGADGFGGGTIYYPRENNTYGAV
AISPGYTGTQASVAWLGKRIASHGFVVITIDTNTTLDQPDSRARQLNAALDYMINDASS
AVRSRIDSSRLAVMGHSMGGGGSLRLASQRPDLKAAIPLTPWHLNKNWSSVRVPTLII
GADLDTIAPVLTHARPFYNSLPTSISKAHLELDGATHFAPNIPNKIIGKYSVAWLKRFVD
NDTRYTQFLCPGPRDGLFGEVGEYRSTCPF

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/053009

A. CLASSIFICATION OF SUBJECT MATTER
INV. A23K1/165 C12N9/16
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A23K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, Sequence Search, EMBASE, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TAKAHASHI-ANDO N ET AL: "A novel lactonohydrolase responsible for the detoxification of zearalenone: enzyme purification and gene cloning", BIOCHEMICAL JOURNAL, THE BIOCHEMICAL SOCIETY, LONDON, GB, vol. 365, 1 July 2002 (2002-07-01), pages 1-6, XP002967677, ISSN: 0264-6021, DOI: 10.1042/BJ20020450 cited in the application page 6, column 1, last paragraph - column 2, paragraph 1	1-14

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
26 April 2012	08/05/2012

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/053009

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2012/053009

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