

POLYPEPTIDE FOR HYDROLYTIC CLEAVAGE OF ZEARALENONE AND/OR ZEARALENONE DERIVATIVES, ISOLATED POLYNUCLEOTIDE THEREOF AS WELL AS A POLYPEPTIDE CONTAINING AN ADDITIVE, USE OF SAME AS WELL AS A PROCESS

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The present invention relates to a polypeptide for hydrolytic cleavage of zearalenone and/or at least one zearalenone derivative, an isolated polynucleotide which codes for such a polypeptide as well as an additive containing such a polypeptide as well as a use of such a polypeptide as well as a method for hydrolytic cleavage of zearalenone and/or at least one zearalenone derivative.

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Mycotoxins are secondary metabolites produced by filamentary fungi. An important representative of mycotoxins is zearalenone (ZEN), which was previously known as F-2 toxin, which is produced by a variety of *Fusarium* fungi and can be found throughout the world. These fungi infest cultivated plants, among others, such as various types of grain, wherein the fungal infestation usually occurs before the harvest when the growth of the fungi and/or the mycotoxin production may take place before storage or may even take place after harvest, either prior to storage or under improper storage conditions. The FAO has estimated that 25 % of agrarian products throughout the world are contaminated with mycotoxins, thus resulting in substantial economic losses. In an international study concluded recently, a total of 23,781 samples were analyzed from January 2009 to December 2011, 81 % of them testing positive for at least one mycotoxin and 45 % testing positive for ZEN. ZEN has been found in all regions of the world and in all types of grain and feed crops tested, such as corn, soy flour, wheat, wheat bran, DDGS (dried distillers grains with solubles) as well as in finished animal feed mixtures with an incidence of up to 100 %.

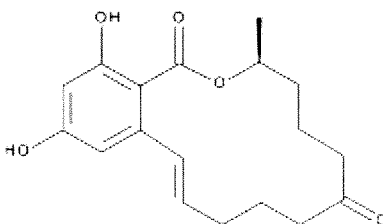
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ZEN is a nonsteroidal estrogenic macrocyclic lactone with the following structural formula, synthesized by way of the polyketide metabolic pathway:

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and its name according to the IUPAC nomenclature is (2E,11S)-15,17-dihydroxy-11-methyl-12-oxabicyclo[12.4.0]octadeca-1(18),2,14,16-tetraene-7,13-dione.

However, a variety of ZEN derivatives also occurs in nature and may be formed by

enzymatic or chemical modifications of ZEN. Examples include glycosidic ZEN conjugates or those containing sulfate, formed by fungi, plants or a mammalian metabolism as well as ZEN metabolites formed in the human or animal organism, among others. ZEN derivatives are understood below to be ZEN conjugates or ZEN metabolites that occur naturally or are synthesized by chemical or biochemical synthesis but in particular α -zearalenol (α -ZEL; (2E,7R,11S)-7,15,17-trihydroxy-11-methyl-12-oxabicyclo[12.4.0]octadeca-1(18),2,14,16-tetraen-13-one), β -zearalenol (β -ZEL; (2E,7S,11S)-7,15,17-trihydroxy-11-methyl-12-oxabicyclo[12.4.0]octadeca-1(18),2,14,16-tetraen-13-one), α -zearalanol (α -ZAL; (7R,11S)-7,15,17-trihydroxy-11-methyl-12-oxabicyclo[12.4.0]octadeca-1(18),14,16-trien-13-one), β -zearalanol (β -ZAL; (7S,11S)-7,15,17-trihydroxy-11-methyl-12-oxabicyclo[12.4.0]octadeca-1(14),15,17-trien-13-one), zearalenone 14-sulfate (Z14S; [(2E,11S)-15-hydroxy-11-methyl-7,13-dioxo-12-oxabicyclo[12.4.0]octadeca-1(18),2,14,16-tetraen-17-yl] hydrogen sulfate), zearalenone-14-glycoside (Z14G; (2E,11S)-15-hydroxy-11-methyl-17-[(3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)-tetrahydropyran-2-yl]oxy-12-oxabicyclo[12.4.0]octadeca-1(18),2,14,16-tetraene-7,13-dione) as well as zearalanone (ZAN; (11S)-15,17-dihydroxy-11-methyl-12-oxabicyclo[12.4.0]octadeca-1(18),14,16-triene-7,13-dione).

ZEN as well as ZEN derivatives, in particular α -ZEL, β -ZEL, Z14S, α -ZAL, β -ZAL, Z14G and ZAN can also be detected in processed foods and animal feed products, such as bread or beer because of their high chemical and physical stability.

ZEN binds to the estrogen receptor and can cause hormonal disruptions, being absorbed immediately after oral ingestion and converted by mammals into the two stereoisomeric metabolites α -ZEL and/or β -ZEL. For example, α -ZEL but also α -ZAL and/or ZAN have a much stronger estrogenic effect than ZEN. Meanwhile, conjugated ZEN derivatives have a lower estrogenic activity than ZEN but ZEN can be released again from these ZEN derivatives in the digestive tract under some circumstances.

Although ZEN has a relatively low acute toxicity and has an oral LD₅₀ of up to 20,000 mg/kg body weight, subacute and/or subchronic toxic effects such as teratogenic, carcinogenic, estrogenic and immunosuppressant effects may occur in animals or humans

with prolonged exposure. Feed contaminated with ZEN leads to developmental disorders in mammalian animals, but pigs, in particular piglets, are extremely sensitive to ZEN. ZEN concentrations of more than 0.5 ppm in feed result in developmental disorders, and concentrations of more than 1.5 ppm in pigs, for example, can result in hyperestrogenicity, and concentrations of 12 ppm ZEN have been blamed for spontaneous abortions in cattle. Since zearalenone is absorbed rapidly through the mucous membranes, in particular through the gastric mucosa as well as the oral mucosa, immediate and quantitative deactivation is essential. ZEN can be detected in blood even 30 minutes after oral administration. In this case, the use of isolated enzymes offers some advantages with respect to microorganisms, such as a higher specific activity or a quicker effect. Because of the harmful effects of ZEN, the European Union has binding upper limits for ZEN in foodstuffs as well as recommendations for upper limits for ZEN in animal feed products (EC No. 1881/2006).

The primary strategy for reducing ZEN contamination of foods and animal feed products is to restrict the growth of fungi, for example, by maintaining "good agricultural practice." This includes, among other things, ensuring that the seed is free of pests and fungal infestation or that agricultural waste products are removed from the field promptly. In addition, fungal growth in the field can be reduced through the use of fungicides. After the harvest, the harvested material should be stored at a residual moisture level of less than 15 % and at a low temperature to prevent the growth of fungi. Likewise, material contaminated by fungal infestation should be removed before further processing. Despite the list of measures, I. Rodrigues and K. Naehrer (2012) have reported that, even in regions with the highest agricultural standards, such as the United States and Central Europe in the years 2009 to 2011, 29 % and 39 % respectively, of the tested corn samples were contaminated with ZEN.

Additional possibilities for removing ZEN from foodstuffs or animal feed products include adsorption and/or transformation of the mycotoxin. This requires that binding of the mycotoxin to the adsorbent must be strong and specific over a wide pH range and must remain stable in the gastrointestinal tract. Although some nonbiological adsorbents such as activated carbon and silicates or synthetic polymers such as cholestyramine can be used efficiently for aflatoxins, their use for other mycotoxins is limited. The main disadvantage of adsorbents is the nonspecific binding of other molecules, which are in some cases essential for nutrition. Biological adsorbents such as yeast or yeast extracts

have also been described in the literature but have a limitation similar to that of nonbiological adsorbents.

5 Detoxification of ZEN by physical and chemical treatments is also limited. ZEN cannot be deactivated effectively by thermal treatment, but the ZEN content can be reduced by 83.9 % by extrusion and treatment with oxidizing agents, for example, for 16 hours at 80°C with 10 % hydrogen peroxide solution. Use of extrusion methods and oxidizing agents such as ozone or hydrogen peroxide in the production of foodstuffs and animal feed products is limited because of the high cost, the loss of quality and in some cases the
10 low efficacy and low specificity.

Biotransformation of ZEN by means of microorganisms such as *Trichosporon mycotoxinivorans*, *Gliocladium roseum* or *Bacillus subtilis* strains and/or enzymes isolated from them such as hydrolases or peroxidases has been described, for example, by E. Vekiru et al. in Appl. and Environ. Microb., 2010, 76, 7, 2353-2359.
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EP 0 938 575 B1 has described ZEN-degrading properties of bacteria of the genus *Rhodococcus* or *Nocardia*, in particular *R. globerulus*, *R. erythropolis* and *N. globerula*.

20 WO 02/076205 describes the ZEN-degrading effect of enzymes isolated from *Gliocladium roseum*, including α,β -hydrolase and zearalenone hydrolase 1 (ZHD1), which catalyze the degradation of ZEN by means of a catalytic triad.

WO 2012/113827 discloses recombinant zonases, namely enzymes that degrade ZEN and remain stable in the gastrointestinal tract. These include microorganisms such as *Thermobifidia fusca*, *Streptomyces exfoliates*, *Acidovorans delafieldii* and *Streptomyces* sp. in particular.
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Polypeptides or enzymes capable of hydrolyzing ZEN and/or at least one ZEN derivative
30 may also be designated as zonases.

The terms used hereinafter are taken from the technical language and each is used in the traditional meanings, unless something to the contrary is indicated. Thus, for example, the term "polynucleotide" relates to all types of genetic material of all lengths and sequences
35 such as single-stranded and double-stranded DNA and RNA molecules, including regulatory elements, structural elements, groups of genes, plasmids, entire genomes and

fragments thereof. The designation "polypeptide" includes proteins such as, for example, enzymes, antibodies as well as polypeptides with up to 500 amino acids, such as, for example, peptide inhibitors, domains of proteins or also short polypeptides with short sequence lengths, for example, less than 10 amino acids, such as receptors, ligands, peptide hormones, tags and the like. The designation "position" in a polynucleotide or polypeptide relates to a single specific base or amino acid in the sequence of the polynucleotide or of the polypeptide.

The present invention is now aimed at making available a polypeptide with which it is possible to rapidly and reliably transform ZEN and/or at least one ZEN derivative into hydrolyzed ZEN and/or hydrolyzed ZEN derivatives. To achieve this object, the present invention is characterized essentially in that the polypeptide is a hydrolase with an amino acid sequence selected from the group of sequence ID numbers 1 to 15 or a functional variation thereof, wherein there is a sequence identity of at least 70 % between the functional variant and at least one of the amino acid sequences.

The term "sequence identity" according to the present invention relates to a percentage sequence identity. For amino acid sequences and nucleotide sequences, the sequence identity can be determined visually, but is preferably calculated by a computer program. The sequence comparison is also carried out within sequence segments, wherein the segment is understood to be a continuous sequence of the reference sequence and preferably comprises a conserved region of the sequence.

In the present case, the sequence identity was determined with the help of the NCBI BLAST program (BLAST = Basic Logic Alignment Search Tool), in particular with BLASTP for polypeptides and BLASTN for polynucleotides, which are made available on the homepage of the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/>). It is thus possible to compare two or more sequences with one another according to the algorithm of Altschul et al., 1997 (Nucleic Acids Res., 25:3389-3402). For this purpose of this invention, the programs were used in the version of 15 May 2013. The basic settings were used as the program settings, but in particular for the amino acid sequence comparison: "max target sequence" = 100; "expected threshold" = 10; "word size" = 3; "matrix" = BLOSUM62; "gap costs" = "existence: 11; extension: 1"; "computational adjustment" = "conditional compositional score matrix adjustment" as well as for the nucleotide sequence comparison word size: 11; expect

value: 10; gap costs: existence = 5, extension = 2; filter = low complexity activated;
match/mismatch scores: 2-3; filter string: L; m.

The terms "functional polypeptide variant" or "functional variant" relate first to "allelic
5 variants" of the polypeptide and to "functional fragments" of the polypeptide and secondly
to "modification" of the polypeptide, wherein the enzymatic function is essentially
unchanged. The term "allelic variant" relates to a polypeptide formed by naturally
occurring mutation(s) in the nucleotide sequence and causing a change in the amino acid
sequence, wherein the enzymatic function thereof is not affected. "Modifications" may be,
10 for example, C- or N-terminal fusions with polypeptides or mutated polypeptides, wherein
mutations can be obtained by substitution, insertion or deletion of at least one amino acid,
in particular by site-directed mutagenesis, i.e., random mutagenesis, recombination and/or
any other protein engineering method. The terms "substitution," "insertion" and "deletion"
are used here in the common meanings in genetic engineering, with which those skilled in
15 the art are familiar. The term "functional fragment" refers to a part or a subsequence of a
polypeptide or a part and/or a subsequence of a functional variant thereof, wherein the
enzymatic function is essentially retained. An enzymatic function is retained in particular
when the enzymatic reaction mechanism remains unchanged, i.e., the mycotoxin is
hydrolyzed in the same location, and the specific residual activity "functional variant"
20 amounts to at least 5 %, preferably at least 10 %, especially at least 10 %, and in
particular at least 50 %, based on the original polypeptide. The polypeptides with the
amino acid sequences having the sequence ID numbers 1 through 15 are functional allelic
variants either of one another or of one and the same enzyme, wherein the sequences
originate from different microorganisms. This is clearly recognizable from the close
25 relationship to one another, measured by means of the percentage sequence identity, as
well as the fact that all polypeptides act on ZEN and ZEN derivatives by means of the
same degradation mechanisms.

Because of the similarity in the amino acid sequences of the polypeptides with the
30 sequence ID numbers 1 through 15 to one another, it is possible that a functional variant
of one of these polypeptides may have a sequence identity of at least 40 %, with more
than one of the claimed polypeptides having the sequence ID numbers 1 through 15.

Through the choice of such an amino acid sequence or a functional variant thereof, a
35 surprisingly fast and complete hydrolysis of ZEN and/or at least one ZEN derivative has
been detected.

As corresponds to a preferred further development of the invention, the polypeptide has an amino acid sequence, which contains at least one conserved amino acid sequence segment or a functional variant thereof, wherein the functional variant of the amino acid sequence segment has a sequence identity of at least 70 %, preferably at least 84 %, more preferably at least 92 % and most preferably at least 98 %, and the at least one conserved amino acid sequence segment is selected from the group of amino acid sequences +24 to +50, +52 to +77, +79 to +87, +89 to +145, +150 to +171, +177 to +193, +223 to +228, +230 to +237, +239 to +247, +249 to +255, +257 to +261, +263 to +270, +272 to +279, +297 to +301, +303 to +313, +24 to 328, +1 to +328 of the sequence having the sequence ID no. 1. Due to the presence of at least one such conserved amino acid sequence segment, it has been possible to make available a polypeptide which also has, in addition to the rapid and complete hydrolysis of ZEN and/or of at least one ZEN derivative, a particularly high activity value in comparison with ZEN degrading polypeptides known previously.

Equally good results have been achieved when according to a further functional development of the invention the functional variant has at least one amino acid modification selected from the group of substitution, deletion and insertion of one or more amino acids.

By further developing the invention in such a way that the polypeptide has a specific activity of at least 0.01 U/mg, preferably at least 0.1 U/mg, in particular at least 1 U/mg; and/or a K_M value of the hydrolytic cleavage of ZEN of at most 50 μM , preferably at most 3.5 μM , in particular at most 0.5 μM ; and/or a k_{cat} value of the hydrolytic cleavage of ZEN of at least 0.05 s^{-1} , preferably at least 0.6 s^{-1} , in particular at least 5 s^{-1} ; and/or a v_{max} value of the hydrolytic cleavage of ZEN of at least $0.00001 \mu\text{M}^{-1} \text{ s}^{-1}$, preferably at least $0.0001 \mu\text{M}^{-1} \text{ s}^{-1}$, in particular at least $0.001 \mu\text{M}^{-1} \text{ s}^{-1}$, then ZEN and/or ZEN derivatives can be hydrolyzed especially rapidly and completely, in particular being detoxified.

According to a preferred embodiment of the invention, the polypeptide contains an amino acid sequence selected from the group of sequence ID numbers 5, 6 and 15 or a functional variant thereof, wherein the functional variant has at least 70 % sequence identity with at least one of the amino acid sequences, and the pH stability of the polypeptide at pH 5.0 amounts to at least 15 %, preferably 50 % and in particular preferably 90 %. It is possible in this way to ensure that the polypeptide zearalenone

and/or at least one zearalenone derivative will be cleaved and/or detoxified even in an acidic medium, such as the mammalian stomach, for example. The pH stability of polypeptides is defined here as the percentage residual activity of the polypeptides at pH 5.0 in relation to the activity at the respective optimum pH.

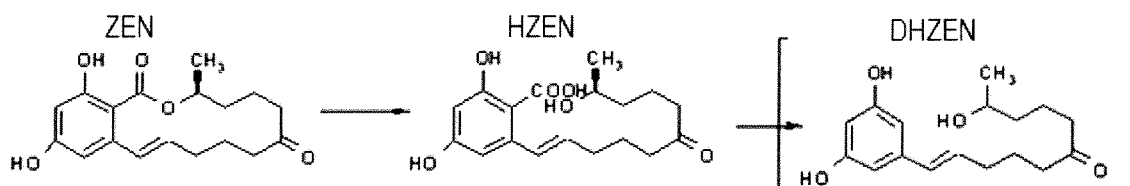
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According to a preferred embodiment of the invention, the polypeptide contains an amino acid sequence selected from the group of sequence ID numbers 1, 5, 6 and 15 or a functional variant thereof, wherein the functional variant has at least 70 % sequence identity with at least one of the amino acid sequences, so that the polypeptide has the highest enzymatic activity in a temperature range between 30°C and 75°C, preferably between 38°C and 55°C, in particular preferably between 38°C and 52°C. This ensures that zearalenone and/or at least one zearalenone derivative is also hydrolyzed and/or detoxified by the polypeptide even at mesophilic temperatures, in particular at the body temperature of humans and farm animals. The temperature at which the polypeptide has the highest enzymatic activity is defined as the optimum temperature of the polypeptide.

According to a preferred embodiment of the invention, the polypeptide has an amino acid sequence selected from the group of sequence ID numbers 1, 5, 6 and 15 or a functional variant thereof, wherein the functional variant has at least 70 % sequence identity with at least one of the amino acid sequences, and the polypeptide is thermally stable up to a temperature of 90°C, preferably 75°C and in particular preferably 60°C. This ensures that the polypeptide and its enzymatic function will remain essentially intact even under elevated temperature stress, such as that which may occur, for example, during shipping in a container or during pelletization of feed. The thermal stability of polypeptides is defined as the temperature at which, after 15 minutes of preliminary incubation, the polypeptide has a 50 % residual activity in comparison with the activity at the respective optimum temperature.

The polypeptide may be selected so that it has an α,β -hydrolase, which is suitable for oxygen-independent and cofactor-free hydrolytic cleavage of the ester group of zearalenone and/or of the ZEN derivatives, which has an amino acid triad that catalyzes the hydrolytic cleavage and consists of serine, an acidic amino acid selected from glutamic acid and aspartic acid, in particular aspartic acid and histidine, and the catalytic triad is, for example, S128, D264 and H303, wherein the positioning relative to the sequence ID no. 1 is shown.

Hydrolysis of ZEN and ZEN derivatives succeeds with any of the polypeptides of the sequence ID numbers 1 to 15 on the ester group of zearalenone or its derivatives according to the following reaction mechanism:



- 5 The hydrolysis of ZEN to form nontoxic hydrolyzed zearalenone (HZEN) and/or hydrolyzed ZEN derivatives takes place by means of polypeptides according to the invention, in particular α,β -hydrolases. The further decarboxylation of HZEN to decarboxylated hydrolyzed ZEN (DHZEN) and/or decarboxylated hydrolyzed ZEN derivatives is usually spontaneous.

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In particular, by means of the aforementioned catalytic triads, it is possible to completely hydrolyze ZEN and ZEN derivatives, wherein the degradation reaction has a good pH stabilizing effect, in particular at a pH in the acidic range.

- 15 Surprisingly it has been found that it is possible to achieve uniformly good results with a polypeptide that contains in a sequence segment consisting of three amino acids before the serine and three amino acids after the serine of the aforementioned catalytic triad, at least one polar amino acid selected from Y, Q, N, T, K, R, E, D and at least one nonpolar amino acid selected from F, M, L, I, V, A, G, P, and it is also possible to improve at least
- 20 one enzyme kinetic parameter.

- In a preferred refinement of the invention, the polypeptide has at least one mutation of the amino acid sequence with respect to the sequence ID no. 1 in at least one of the following positions: 22, 23, 25, 26, 27, 29, 31, 32, 35, 37, 42, 43, 46, 51, 53, 54, 57, 60, 69, 72, 73,
- 25 78, 80, 84, 88, 95, 97, 99, 114, 118, 119, 123, 132, 141, 146, 148, 149, 154, 163, 164, 165, 169, 170, 172, 176, 180, 182, 183, 190, 191, 194, 196, 197, 198, 201, 204, 205, 206, 207, 208, 209, 210, 212, 213, 214, 216, 217, 220, 221, 222, 229, 231, 233, 238, 240, 244, 245, 246, 248, 249, 251, 254, 256, 260, 262, 263, 266, 269, 271, 277, 280, 281, 282, 283, 284, 285, 286, 287, 292, 296, 298, 302, 307, 308, 309, 311, 314, 317, 319, 321, 323, 325
- 30 and 326. These positions are derived from the sequence differences between the polypeptide with the sequence ID no. 1 and the polypeptides having the sequence ID numbers 2 to 6, which are especially active and have a high degree of identity with this

sequence. If the polypeptide with sequence ID no. 1 is modified in at least one of these positions, so that the amino acid variants of sequence ID numbers 2 through 6 can be taken over in this position, it is possible to show that these positions have a significant influence on the enzyme kinetic parameters of the polypeptide and that combinations of
5 the sequence ID no. 1 with sequence ID numbers 2 through 6 also having a high degree of sequence identity in addition will lead to higher activities.

According to one refinement of the invention, the polypeptide has at least one mutation selected from the group comprising: D22A, S23Q, S23L, N25D, I26V, F27Y, F27H, S29P,
10 R31A, F32Y, R35K, R35Q, V37A, V42I, V43T, F46Y, S51E, S51D, D53G, N54M, N54R, L57V, L60I, S69G, P72E, V73A, A78S, N80H, F84Y, I88L, T95S, T97A, R99K, I114M, I118V, K119R, V123I, L132V, A141S, I146V, I146L, A148G, A149V, A154P, P163T, A164T, Y165C, Y165H, V169I, L170R, A172G, A176M, A176V, Y180F, D182T, F183Y, I190V, G191S, K194T, K194E, F196Y, V197C, V197R, E198R, E198S, K201D, K201G,
15 P204S, P204A, A205S, K206P, A207M, M208A, Q209R, L210A, L210S, ΔP212, T213V, P214A, E216T, E216G, A217I, N220H, L221M, K222R, K222Q, G229A, A231V, F233W, F233Y, F233H, A238G, H240N, H240S, D244E, R245Q, M246L, S248T, S248N, S248G, Q249R, K251N, I254V, I256L, A260M, T262D, T262G, I263T, E266D, E269H, E269N, L271V, L277E, E280A, E280L, H281R, H281Q, A282V, Q283R, D284L, D284R, I285L,
20 I286M, R287E, R287D, R292K, R292T, Q296A, Q296E, H298V, L302S, L307Q, F308S, D309A, A311P, A314V, L317F, S319Q, S319P, S319R, S321A, S321T, T323A, P325A, A326P in the amino acid sequence with respect to sequence ID no. 1. With such a polypeptide, it is possible to completely hydrolyze ZEN within a short period of time, in particular to detoxify it, wherein the specific activity of the polypeptide amounts to at least
25 6.00 U/mg, preferably at least 7.00 U/mg, in particular at least 8.00 U/mg. The unit "U" or also "unit" is a measure of the absolute catalytic activity and is defined by the hydrolysis of 1 μmol ZEN per minute at 32°C in 50 mM Tris-HCl buffer (pH 8.2), wherein "catalytic activity" is understood to refer to the enzymatic conversion of a substrate under defined reaction conditions, and "specific activity" is understood to refer to the ratio of the catalytic
30 activity and the polypeptide mass concentration (mass per unit of volume).

If, the polypeptide is embodied so that at least one of the following amino acid motifs with a sequence having sequence ID numbers 32 to 50 is contained in it, it is then possible to make available polypeptides having a specific activity of at least 17.00 U/mg, preferably at
35 least 8.00 U/mg. It has surprisingly been found that when at least one of the following amino acid motifs with a sequence having the sequence ID numbers 51 to 58 is contained

in it, the enzymatic activity of the polypeptide is increased further, for example, in comparison with a motif containing seven amino acids. An even higher specific activity is achieved when at least one of the following amino acid motifs having the sequence having the sequence ID numbers 59 to 69 is contained in it.

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According to a further functional development of the invention, the polypeptide may contains at least one conservative amino acid substitution in at least one position, where the conservative amino acid substitution is selected from substitutions of G to A; or A to G, S; or V to I, L, A, T, S; or I to V, L, M; or L to I, M, V; or M to L, I, V; or P to A, S, N; or F to Y, W, H; or Y to F, W, H; or W to Y, F, H; or R to K, E, D; or K to R, E, D; or H to Q, N, S; or D to N, E, K, R, Q; or E to Q, D, K, R, N; or S to T, A; or T to S, V, A; or C to S, T, A; or N to D, Q, H, S; or Q to E, N, H, K, R, wherein the designation "conservative amino acid substitution" relates to the substitution of amino acids by other amino acids regarded by those skilled in the art as being conservative, i.e., having similar specific properties. Such specific properties include, for example, the size, polarity, hydrophobicity, charge or pKs value of the amino acid. A conservative mutation, for example, is understood to be a substitution of one acidic amino acid for another acidic amino acid, a basic amino acid for another basic amino acid or a polar amino acid for another polar amino acid.

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With such conservative amino acid substitutions, it is possible to produce functional polypeptide variants whose specific activity is approximately the same in comparison with the parental polypeptide but is preferably increased by at least 0.1 U/mg.

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The present invention is additionally aimed at making available an isolated polynucleotide with which it is possible to synthesize a polypeptide for rapid and reliable hydrolytic cleavage of ZEN and/or of at least one ZEN derivative.

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To achieve this object, the invention is characterized in that the isolated polynucleotide may have a nucleotide sequence that codes for a polypeptide, wherein the polypeptide has a zearalenone and/or the property of hydrolyzing at least one zearalenone derivative, and the nucleotide sequence codes for at least one polypeptide according to any of the claims 1 to 11 and/or the nucleotide sequence has a degree of sequence identity of at least 40 % with a nucleotide sequence selected from the group of sequence ID numbers 16 to 31 and/or the nucleotide sequence hybridizes under moderate stringency conditions with at least one nucleotide sequence selected from the group of sequence ID numbers 16 to 31 and/or with a subsequence thereof with at least 200 nucleotides, in particular at

least 100 nucleotides and/or with a complementary strand of the nucleotide sequence or subsequences thereof.

Nucleotide sequences to be expressed, in particular their triplets (codons) are usually altered depending on the host cell so that the codon bias is optimized according to the host cell. This results in the fact that even polynucleotides having a degree of sequence identity of far less than 80 % but even less than 70 % or less than 60 % can code for the same polypeptide. The sequence comparison for determining the degree of sequence identity must also be performed within sequence segments, wherein one section is to be understood as a continuous sequence of the reference sequence. The length of the sequence segments for nucleotide sequences is normally 15 to 600.

With the help of the present isolated nucleotide sequences or sequence segments, it is possible to generate nucleic acid probes having a length of usually at least 15, 30 or 40 nucleotides. With such probes, which are typically also labeled, e.g., by ^3H , ^{32}P , ^{35}S , biotin or avidine, it is possible, by using standard methods, to identify nucleotide sequences that code for polypeptides with the property of degrading ZEN and/or ZEN derivatives. For example, DNA, RNA or cDNA from individual microorganisms, genomic DNA libraries or cDNA libraries can be used as the starting material for identification of such sequences.

For nucleotide sequences and/or nucleotide probes with a length of at least 100 nucleotides, moderate stringency conditions are defined as prehybridization and hybridization at 42°C in Na-EDTA buffer provided with 5× NaCl (SPE, 0.9M NaCl, 60 mM NaH_2PO_4 , 6 mM EDTA) containing 0.3 % sodium dodecyl sulfate (SDS), 200 µg/mL sheared and denatured salmon sperm DNA and 35 % formamide followed by standard Southern Blot conditions, wherein the carrier material is washed three times at the end for 15 minutes each with 2× sodium chloride citrate buffer (SSC, 300mM NaCl and 30 mM trisodium citrate, 0.2 % SDS) at 55°C.

For nucleotide sequences and/or nucleotide probes with a length of 15 nucleotides to 100 nucleotides, moderate stringency conditions are defined as prehybridization and hybridization in buffer consisting of 0.9M NaCl, 0.09M Tris-HCl pH = 7.6, 6 mM EDTA, 0.5 % NP-40, 1× Denhardt's solution, 1 mM sodium pyrophosphate, 1 mM sodium dihydrogen phosphate, 0.1 mM ATP and 0.2 mg/mL yeast RNA, wherein prehybridization and hybridization are performed at a temperature 5°C to 10°C below the calculated melting point (T_m), where T_m is determined by calculation according to Bolton and

McCarthy (1962, Proceedings of the National Academy of Sciences USA, 48:1390).

Following this, the experiment is continued under standard Southern Blot conditions (J. Sambrook, E. F. Fritsch and T. Maniatis, 1989, Molecular Cloning, A Laboratory Manual, 2nd edition, Cold Spring Harbor, New York). The carrier material is washed at the end
5 once for 15 minutes with 6× SCC buffer [sic; SSC buffer] containing 0.1 % SDS and twice for 15 minutes with 6× SSC buffer each at 5°C to 10°C under the calculated T_m.

The present invention is additionally aimed at making available an additive with which it is possible to achieve a rapid and reliable hydrolytic cleavage of ZEN and/or at least one
10 ZEN derivative in a defined or complex matrix, such as, for example, in food or animal feed products.

To achieve this goal, an additive that hydrolytically cleaves a zearalenone and/or at least zearalenone derivative is available for animal feed products for pigs, poultry or
15 aquaculture, for foodstuffs or DDGS (distillers dried grain and solubles), wherein the additive contains at least one polypeptide having an amino acid sequence selected from the group of sequence ID numbers 1 to 15 or a functional variant thereof, wherein the sequence identity between the functional variant and at least one of the amino acid sequences amounts to at least 70 %, and auxiliary substances are also present.

20 With such an additive, the biochemical conversion of ZEN and/or at least one ZEN derivative to hydrolyzed ZEN and/or hydrolyzed ZEN derivative is possible. This additive can also be used, for example, for stereoselective hydrolysis of ZEN and/or ZEN derivatives in industrial processes.

25 In a preferred refinement of the invention, the additive is embodied so that the auxiliary substances are selected from at least one inert carrier as well as optionally additional ingredients, such as vitamins and/or minerals and/or enzymes and/or additional components for detoxification of mycotoxins. Due to the use of such an additive, for
30 example, in foodstuffs or animal feed products, it is possible to ensure that any amounts of ZEN and/or ZEN derivatives that might be present are reliably hydrolyzed, in particular being detoxified to the extent that they will have no harmful effect on the organism of the subject consuming this foodstuff or animal feed product.

35 A polypeptide according to the invention here may also be present in an enzyme preparation, which additionally contains at least one enzyme in addition to at least one

polypeptide according to the invention, such that the enzyme takes part in the degradation of proteins, for example, such as proteases, or plays a role in the metabolism of starch or fiber or fat or glycogen, such as, for example, amylase, cellulase or glucanases as well as, for example, hydrolases, lipolytic enzymes, mannosidase, oxidases, oxidoreductases, phytases, xylanases and/or combinations thereof.

Additional fields of use of the invention include enzyme preparations, which, in addition to at least one polypeptide according to the invention, also contain at least one component for detoxification of mycotoxins, such as a mycotoxin-degrading enzyme, for example, aflatoxin oxidase, ergotamine hydrolases, ergotamine amidases, zearalenone esterases, zearalenone lactonases, ochratoxin amidases, fumonisin carboxyl esterases, fumonisin aminotransferases, aminopolyol aminooxidases, deoxynivalenol epoxide hydrolases and/or at least one mycotoxin-degrading microorganism, such as *Bacillus subtilis* and/or at least one mycotoxin-binding component, for example, microbial cell walls or inorganic materials such as bentonites.

According to one particularly preferred refinement of the invention, at least one polypeptide is present in the additive in a concentration of at most 10,000 U/g, preferably at most 1000 U/g, more preferably at most 100 U/g and most preferably at most 10 U/g, so that it is possible to convert ZEN and/or ZEN derivatives rapidly and in particular to do so already before they are absorbed by the body of a subject, in particular a mammal consuming a contaminated foodstuff or animal feed product, converting them into nontoxic or less toxic metabolites, in particular HZEN and DHZEN.

According to a refinement of the invention, the polypeptide is present in encapsulated or coated form, wherein standard methods such as those described in WO 92/12645 can be used for the encapsulation or coating. By encapsulation and/or coating, it is possible to transport the polypeptide without any change, in particular without degradation or damage, to its site of use, so that only after the protective shell has been dissolved in the digestive tract of animals, for example, does the polypeptide begin to act so that an even more targeted, rapid and complete degradation of ZEN and/or ZEN derivatives can be achieved even in the acidic protease-rich and anaerobic medium. In addition, it is also possible through encapsulation or coating to increase the thermal stability of the polypeptides in the additive.

The present invention is additionally aimed at use of an additive containing at least one polypeptide having an amino acid sequence of sequence ID numbers 1 to 15 or a functional variant thereof, wherein the sequence identity between the functional variant and at least one of the amino acid sequences amounts to at least 70 % for hydrolytic cleavage of zearalenone and/or at least one zearalenone derivative in animal feed products, in particular for pigs, poultry and agriculture, in foodstuffs or in distillers dried grain and solubles. Through the use of the additive according to the invention, it is possible to hydrolyze and/or detoxify the ZEN and/or ZEN derivatives contained in the foodstuff or animal feed product and/or distillers dried grain and solubles, wherein such a detoxification is possible even with polypeptide concentrations of approximately 1 U/g contaminated foodstuff or animal feed product.

The present invention is additionally aimed at making available a method with which a rapid and reliable hydrolytic cleavage of ZEN and/or at least one ZEN derivative is made possible.

To achieve this goal, this method is carried out in such a way that zearalenone and/or at least one zearalenone derivative having an amino acid sequence of sequence ID numbers 1 or a functional variant thereof is hydrolyzed, wherein the sequence identity between the functional variant and at least one of the amino acid sequences amounts to at least 70 %.

According to one refinement of the invention the method is carried out in such a way that the polypeptide therein is used in an additive corresponding to this invention.

According to another preferred refinement, the method is carried out in such a way that the polypeptide or the additive therein is mixed with a foodstuff or animal feed product contaminated with zearalenone and/or with at least one zearalenone derivative; the contaminated foodstuff or animal feed product is brought in contact with moisture and the polypeptide or the additive hydrolyzes the zearalenone and/or at least one zearalenone derivative contained in the contaminated foodstuff or animal feed product. In the case of moist foodstuffs or animal feed products, such as mash or slurries, the hydrolysis of the zearalenone and/or of at least one zearalenone derivative will take place in the moist foodstuff or animal feed product before oral consumption. Due to this method, it is possible to ensure that the harmful effects of zearalenone and zearalenone derivatives on humans and animals will be largely eliminated. Moisture here is understood to refer to the presence of water or aqueous liquids, which also include, for example, saliva or other

liquids present in the digestive tract. The digestive tract is defined as the oral cavity, the pharynx (throat), the esophagus and the gastrointestinal tract or equivalents thereof, wherein there may be different designations for animals and/or individual components may not occur in the digestive tract of animals.

5

The method according to the invention may also be carried out in such a way that the foodstuff or animal feed product is pelletized before oral consumption.

10

According to one refinement of the invention, the method is carried out so that at least 70 %, preferably at least 80 % in particular at least 90 % of the zearalenone and/or at least one zearalenone derivative is hydrolyzed. Therefore, subacute and/or chronic toxic effects such as teratogenic, carcinogenic, estrogenic and immunosuppressant effects in animals or humans, for example, can be suppressed.

15

The invention is explained in greater detail below on the basis of exemplary embodiments as well as drawings, in which:

20

Figure 1 shows the degradation of ZEN and the increase in the metabolites HZEN and DHZEN over time for the polypeptide having the sequence ID no. 1, wherein the polypeptide in Figure 1A has not been tagged, in Figure 1B the polypeptide has a C-terminal 6xHis tag, and in Figure 1C the polypeptide has an N-terminal 6xHis tag,

Figure 2 shows the Michaelis-Menten kinetics of the polypeptide with sequence ID no. 1,

25

Figure 3 shows the degradation of ZEN and the increase in metabolites HZEN and DHZEN over time, due to purified polypeptides having the sequence ID numbers 1 (Figure 3A), 2 (Figure 3B), [5?] (Figure 3C), 6 (Figure 3D), 7 (Figure 3E), 9 (Figure 3F), [11?] (Figure 3G), [12?] (Figure 3H) and 15 (Figure 3I), wherein all the sequences have a C-terminal 6xHis tag.

30

Example 1: Modification, cloning and expression of polynucleotides that code for polypeptides which are capable of hydrolytic cleavage of ZEN and/or at least one ZEN derivative

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Amino acid substitutions, insertions or deletions were performed by mutation of the nucleotide sequences by means of PCR using the "quick change site-directed

mutagenesis kits" (Stratagene) according to the instructions. As an alternative, complete nucleotide sequences were also ordered (GeneArt). The nucleotide sequences generated by means of PCR mutagenesis and/or ordered from GeneArt optionally also contained a C- or N-terminal 6xHis tag on an amino acid level and were integrated by means of standard methods into expression vectors for expression in *E. coli* or *P. pastoris*, transformed in *E. coli* or *P. pastoris* and expressed in *E. coli* and *P. pastoris* (J. M. Cregg, Pichia Protocols, second edition, ISBN-10: 1588294293, 2007; J. Sambrook et al., 2012, Molecular Cloning, A Laboratory Manual, 4th edition, Cold Spring Harbor), wherein any other suitable host cell may also be used for this task.

The designation "expression vector" relates to a DNA construct that is capable of expressing a gene *in vivo* or *in vitro*. In particular this is understood to refer to DNA constructs that are suitable for transferring the polypeptide coding nucleotide sequence into the host cell to integrate into the genome there or to be present freely in the extrachromosomal space and to express the polypeptide coding nucleotide sequence intracellularly and optionally also to remove the polypeptide from the cell.

The designation "host cell" refers to all cells containing either a nucleotide sequence to be expressed or an expression vector and being capable of synthesizing a polypeptide according to the invention. In particular this is understood to include prokaryotic and/or eukaryotic cells, preferably *P. pastoris*, *E. coli*, *Bacillus subtilis*, *Streptomyces*, *Hansenula*, *Trichoderma*, *Lactobacillus*, *Aspergillus*, plant cells and/or spores of *Bacillus*, *Trichoderma* or *Aspergillus*.

The soluble cell lysate in the case of *E. coli* and/or the culture supernatant in the case of *P. pastoris* was/were used for determination of the catalytic properties of the polypeptides. To determine the K_M value, v_{max} , k_{cat} and the specific activity, the polypeptides were selectively enriched chromatographically by standard methods over nickel-Sepharose columns. The determination of the protein concentration was performed by means of standard methods, either being calculated by the BCA method (Pierce BCA Protein Assay KitProd #23225) or preferably photometrically with the specific extinction coefficients for the respective proteins that are available online with the ProtParam program at <http://web.expasy.org/protparam> (Gasteiger E. et al.; *Protein Identification and Analysis Tools on the ExPASy Server*, in John M. Walker (ed): The Proteomics Protocols Handbook, Humana Press, 2005, pp. 571-607).

Example 2: Determination of the sequence identity and the conserved amino acid sequence segments

The determination of the percentage sequence identity based on the total polypeptide length of the polypeptides with eh amino acid sequences having the sequence ID numbers 1 to 15 relative to one another (Table 1) was performed with the help of the BLAST program (Basic Local Alignment Search Tool), in particular with BLASTP, which can be used at homepage of the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov/>). It is thus possible to compare two or more sequences with one another according to the algorithm of Altschul et al., 1997 (Nucleic Acids Res. (1997), 25:3389-3402). The basic settings were used as the program settings in particular. However: "max target sequence" = 100; expected threshold" = 10; "word size" = 3; "matrix" = BLOSUM62; "gap costs" = "existence: 11; extension: 1"; "computational adjustment" = "conditional compositional score matrix adjustment."

To determine the conserved amino acid sequence segments, the polypeptides having sequence ID numbers 1 to 6, which have a sequence identity of at least 70 % with one another, were compared with the help of the COBALT software (J. S. Papadopoulos and R. Agarwala, 2007, COBALT: Constraint-Based Alignment Tool for Multiple Protein Sequences, Bioinformatics 23:1073-79) while using the standard parameters, in particular the parameters ("gap penalties": -11, -1; "end-gap penalties": -5, -1; "use RPS BLAST": on; "Blast E-value": 0.003; "find conserved columns and recompute": on; "use query clusters": on; "word size": 4; "may cluster distance": 0,8; "alphabet": regular; "homology conversation setting": 3 bits). The result of this analysis represents the conserved amino acids. The following ranges of at least five successive conserved amino acids were defined as the conserved amino acid sequence segments, namely with respect to the segment having the sequence ID no. 1, the segments A from position +24 to position +50, B from position +52 to position +77, C from position +79 to position +87, D from position +89 to position +145, E from position +150 to position +171, F from position +177 to position +193, G from position +223 to position +228, H from position +230 to position +237, I from position +239 to position +247, J from position +249 to position +255, K from position +257 to position +261, L from position +263 to position +270, M from position +272 to position +279, N from position +297 to position +301 and O from position +303 to position +313.

The determinations of the percentage sequence identity of the polypeptides to one another and of the conserved amino acid sequence segments of the individual polypeptides relative to the conserved amino acid sequence segments of the sequence having the sequence ID no. 1 were formed as described above. The results are presented in Tables 1 and 2.

Table 1: Percentage sequence identity of the polypeptides to one another.

	SEQ ID No. 1	SEQ ID No. 2	SEQ ID No. 3	SEQ ID No. 4	SEQ ID No. 5	SEQ ID No. 6	SEQ ID No. 7
SEQ ID No. 1	-	70 %	71 %	71 %	71 %	71 %	64 %
SEQ ID No. 2	70 %	-	81 %	83 %	81 %	83 %	63 %
SEQ ID No. 3	71 %	81 %	-	95 %	99 %	92 %	60 %
SEQ ID No. 4	71 %	83 %	95 %	-	95 %	95 %	60 %
SEQ ID No. 5	71 %	81 %	99 %	95 %	-	93 %	60 %
SEQ ID No. 6	71 %	83 %	92 %	95 %	93 %	-	61 %
SEQ ID No. 7	64 %	63 %	60 %	60 %	60 %	61 %	-
SEQ ID No. 8	57 %	54 %	54 %	53 %	53 %	53 %	53 %
SEQ ID No. 9	50 %	50 %	53 %	53 %	53 %	55 %	51 %
SEQ ID No. 10	55 %	52 %	55 %	54 %	55 %	53 %	52 %
SEQ ID No. 11	53 %	51 %	53 %	51 %	51 %	52 %	54 %
SEQ ID No. 12	50 %	49 %	50 %	50 %	50 %	49 %	51 %
SEQ ID No. 13	55 %	49 %	51 %	51 %	51 %	52 %	54 %

SEQ ID No. 14	73 %	65 %	69 %	70 %	69 %	68 %	80 %
SEQ ID No. 15	79 %	68 %	71 %	71 %	71 %	72 %	63 %

	SEQ ID No. 8	SEQ ID No. 9	SEQ ID No. 10	SEQ ID No. 11	SEQ ID No. 12	SEQ ID No. 13	SEQ ID No. 14	SEQ ID No. 15
SEQ ID No. 1	57 %	50 %	55 %	53 %	50 %	55 %	73 %	79 %
SEQ ID No. 2	54 %	50 %	52 %	51 %	49 %	49 %	65 %	68 %
SEQ ID No. 3	54 %	53 %	55 %	53 %	50 %	51 %	69 %	71 %
SEQ ID No. 4	53 %	53 %	54 %	51 %	50 %	51 %	70 %	71 %
SEQ ID No. 5	53 %	53 %	55 %	51 %	50 %	51 %	69 %	71 %
SEQ ID No. 6	53 %	55 %	53 %	52 %	49 %	52 %	68 %	72 %
SEQ ID No. 7	53 %	51 %	52 %	54 %	51 %	54 %	80 %	63 %
SEQ ID No. 8	-	50 %	49 %	51 %	49 %	48 %	83 %	51 %
SEQ ID No. 9	50 %	-	51 %	52 %	69 %	51 %	67 %	51 %
SEQ ID No. 10	49 %	51 %	-	76 %	52 %	52 %	63 %	56 %
SEQ ID No. 11	41 %	50 %	76 %	-	52 %	51 %	58 %	52 %
SEQ ID No. 12	49 %	52 %	52 %	52 %	-	49 %	71 %	51 %
SEQ ID No. 13	48 %	51 %	52 %	51 %	49 %	-	54 %	53 %
SEQ ID No. 14	83 %	67 %	63 %	58 %	71 %	55 %	-	72 %

SEQ ID No. 15	51 %	51 %	56 %	52 %	51 %	53 %	72 %	-
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Table 2: Percentage sequence identity of the conserved amino acid sequence segments A to O.

	Sequence identity relative to the sequence ID no. 1					
Polypeptide	Segment A	Segment B	Segment C	Segment D	Segment E	Segment F
SEQ ID No. 1	100 %	100 %	100 %	100 %	100 %	100 %
SEQ ID No. 2	59.6 %	76.9 %	88.9 %	87.7 %	77.3 %	76.5 %
SEQ ID No. 3	63.0 %	76.9 %	77.8 %	89.5 %	86.4 %	76.5 %
SEQ ID No. 4	63.0 %	80.8 %	77.8 %	91.2 %	86.4 %	76.5 %
SEQ ID No. 5	63.0 %	76.9 %	77.8 %	87.7 %	86.4 %	76.5 %
SEQ ID No. 6	63.0 %	80.8 %	77.8 %	91.2 %	86.4 %	76.5 %
SEQ ID No. 7	44.7 %	69.2 %	77.8 %	78.9 %	68.2 %	64.7 %
SEQ ID No. 8	40.7 %	50.0 %	66.7 %	82.5 %	59.1 %	64.7 %
SEQ ID No. 9	51.9 %	57.7 %	55.6 %	73.7 %	45.5 %	58.8 %
SEQ ID No. 10	44.4 %	61.5 %	77.8 %	75.4 %	47.8 %	76.5 %
SEQ ID No. 11	44.4 %	50.0 %	66.7 %	71.9 %	43.5 %	58.8 %
SEQ ID No. 12	51.9 %	53.8 %	55.6 %	71.9 %	50.0 %	58.8 %
SEQ ID No. 13	18.5 %	61.5 %	55.6 %	77.2 %	54.5 %	52.9 %
SEQ ID No. 14	55.6 %	69.2 %	77.8 %	84.2 %	54.5 %	52.9 %
SEQ ID No. 15	74.1 %	86.7 %	88.9 %	89.0 %	77.3 %	88.2 %

	Sequence identity relative to the SEQ ID No. 1					
Polypeptide	Segment G	Segment H	Segment I	Segment J	Segment K	Segment L
SEQ ID No. 1	100 %	100 %	100 %	100 %	100 %	100 %
SEQ ID No. 2	100 %	87.5 %	66.7 %	85.7 %	80.0 %	75.0 %
SEQ ID No. 3	100 %	87.5 %	77.8 %	57.1 %	80.0 %	75.0 %
SEQ ID No. 4	100 %	87.5 %	77.8 %	57.1 %	80.0 %	75.0 %

SEQ ID No. 5	100 %	87.5 %	77.8 %	57.1 %	80.0 %	75.0 %
SEQ ID No. 6	100 %	75.0 %	77.8 %	85.7 %	80.0 %	87.5 %
SEQ ID No. 7	100 %	87.5 %	66.7 %	71.4 %	100 %	50.0 %
SEQ ID No. 8	100 %	62.5 %	44.4 %	57.1 %	80.0 %	62.5 %
SEQ ID No. 9	100 %	12.5 %	44.4 %	42.9 %	60.0 %	62.5 %
SEQ ID No. 10	100 %	62.5 %	55.6 %	71.4 %	80.0 %	50.0 %
SEQ ID No. 11	100 %	50.0 %	55.6 %	57.1 %	80.0 %	50.0 %
SEQ ID No. 12	100 %	12.5 %	22.2 %	57.1 %	80.0 %	52.5 %
SEQ ID No. 13	100 %	50.0 %	44.4 %	57.1 %	80.0 %	75.0 %
SEQ ID No. 14	0 %	8.3 %	0 %	14.3 %	0 %	25.0 %
SEQ ID No. 15	100 %	87.5 %	100 %	85.7 %	100 %	75.0 %

	Sequence identity relative to the SEQ ID No. 1		
Polypeptide	Segment M	Segment N	Segment O
SEQ ID No. 1	100 %	100 %	100 %
SEQ ID No. 2	87.5 %	80.0 %	81.8 %
SEQ ID No. 3	87.0 %	80.0 %	81.8 %
SEQ ID No. 4	87.5 %	80.0 %	81.8 %
SEQ ID No. 5	87.5 %	80.0 %	81.8 %
SEQ ID No. 6	87.5 %	80.0 %	72.7 %
SEQ ID No. 7	75.0 %	40.0 %	36.4 %
SEQ ID No. 8	75.0 %	60.0 %	54.5 %
SEQ ID No. 9	62.5 %	40.0 %	54.5 %
SEQ ID No. 10	62.5 %	40.0 %	54.5 %
SEQ ID No. 11	75.0 %	40.0 %	54.5 %
SEQ ID No. 12	100 %	40.0 %	54.5 %
SEQ ID No. 13	50.0 %	40.0 %	63.6 %
SEQ ID No. 14	6.2 %	0 %	0 %
SEQ ID No. 15	87.5 %	80.0 %	63.6 %

Example 3: Hydrolysis of ZEN by polypeptides in cell lysates

To determine their ability to degrade ZEN into the nontoxic or less toxic metabolites HZEN and DHZEN, the polypeptide with the sequence ID no. 1, coded by the nucleotide
5 sequence having the sequence ID no. 17 was synthesized as such and with a C-terminal and/or N-terminal 6xHis tag in *E. coli* as described in example 1. The polypeptides with the amino acid sequences having the sequence ID numbers 2 to 15 which were coded by the nucleotide sequences having the sequence ID numbers 18 to 31, were labeled with 6xHis exclusively at the C-terminus. 100 mL portions of an *E. coli* culture having an optical
10 density (OD 600 nm) of 2.0-2.5 were harvested by centrifugation at 4°C and resuspended in 20 mL Brunner mineral medium (DSMZ microorganisms medium number 462, 2012). The cell suspensions were lysed by treating three times with a French press at 20,000 psi. The resulting cell lysates were used in a 1:10, 1:100 or 1:1000 dilution prepared in Brunner mineral medium including 0.1 mg/mL BSA (bovine serum albumin). For the ZEN
15 degradation experiments, 9.9 mL Brunner mineral medium was used, including 0.1 mg/mL BSA, 0.1 mL dilute cell lysate and 31 µL ZEN substrate stock solution. On the whole, the cell lysates were thus diluted 1:1000, 1:10,000 and/or 1:100,000. The ZEN substrate stock solution used was a 2.08 mM ZEN solution (40 vol % CAN + 60 vol % H₂O). To prepare this solution, ZEN in crystalline form (Biopure Standard from Romer Labs, article no.
20 001109, purity at least 98 %) was weighed and dissolved accordingly. Each degradation batch was carried out in 25 mL glass vials and incubated at 25°C and 100 rpm for a total of 120 hours with agitation. At the times 0, 0.5, 1, 2, 5, 24, 47, 72 and 120 h, a sample of 1 mL was taken each time, the polypeptides were heat inactivated for 10 minutes at 99°C and stored at -20°C. After thawing the sample, the insoluble constituents were separated
25 by centrifugation. ZEN, HZEN and DHZEN were analyzed by means of LC/MS/MS. To do so, the metabolites were separated chromatographically on a Phenomenex Luna C18(2) column having the dimensions 250 mm × 3 mm and a particle size of 5 µm, using as the mobile phase an acetonitrile-water mixture with a formic acid concentration of 1 mL/L. The UV signal at 270 nm was recorded using electrospray ionization (ESI) as the ionizing
30 source. ZEN, HZEN and DHZEN were quantified by means of QTrap/LC/MS/MS (triple quadrupole, Applied Biosystems) in the enhanced mode. After 24 hours at the latest, substantial amounts of ZEN could not be detected any more in any of the batches. Most of the ZEN, i.e., more than 80 %, was converted into HZEN or DHZEN.

Figure 1 shows the degradation of ZEN over time and the increase in HZEN as well as
35 DHZEN for a 1:10,000 diluted cell lysate solution as an example for untagged (Figure 1A) as well as for C-terminal 6xHis tagged (Figure 1B) and N-terminal 6xHis tagged (Figure

1C) polypeptide with the sequence ID no. 1. It can be seen here clearly that 1) the reaction of ZEN takes place directly and completely because almost no ZEN could be detected any longer in the first sample (0 h), which was taken immediately after the start of the experiment, and 2) no mentionable losses of activity occurred as a result of
5 attaching a tag, whether C-terminal or N-terminal.

Example 4: Hydrolysis of ZEN derivatives by polypeptides in cell lysates

To determine the capability of polypeptides to also transform ZEN derivatives, in addition
10 to ZEN, into nontoxic and/or less toxic metabolites, the polypeptides having the sequence ID numbers 1 to 15 were prepared as described in Example 3 with C-terminal His tag and the respective synthetic nucleotide sequences with the sequences having sequence ID numbers 17 to 31 were used as the cell lysates in degradation 15.

15 The degradation experiments were performed as described in Example 3, where each polypeptide was tested with each ZEN derivative selected from the group comprised of α -ZEL, β -ZEL, α -ZAL, β -ZAL, Z14G, Z14S and ZAN. The cell lysates were used in a total dilution of 1:10,000. Instead of a 2.08 mM ZEN solution (40 vol % CAN + 60 vol % H₂O), equimolar, i.e., 2.08 mM solutions of the ZEN derivatives were used as the substrate
20 stock solution. α -ZEL, β -ZEL, α -ZAL, β -ZAL and ZAN were obtained from Sigma and used as standards for the analysis. Z14G and Z14S were prepared in a purity of at least 90 % according to the methods such as those described by P. Krenn et al., 2007 (Mycotoxin Research, 23, 4, 180-184) and M. Sulyok et al., 2007 (Anal. Bioanal. Chem. 289, 1505-1523) and used as standards for the analysis. Another difference in comparison with
25 Example 3 is that only one sample was taken, namely after 24 hours. The reduction in concentration of the ZEN derivatives during the degradation experiment was quantified by means of LC/MS/MS. α -ZEL, β -ZEL, Z14G and Z14S were measured by the method of M. Sulyok et al. (2010, Food Chemistry, 119, 408-416); α -ZAL, β -ZAL and ZAN were
30 measured by the method of P. Songsermaskul et al. (2011, J. of Animal Physiol. and Animal Nutr., 97, 155-161). It was surprisingly found that only 0 to max. 13 % of the starting amounts of the ZEN derivatives was present after 24 hours of incubation in all the degradation experiments.

Example 5: Specific activity and enzyme kinetic parameters of the polypeptides as well as
35 variants thereof

The specific activity of the polypeptides and variants thereof was determined photometrically, wherein all the polypeptides used had a C-terminal 6xHis tag. The preparation, enrichment and purification of the polypeptides and/or variants thereof were performed as described in example 1. Degradation of ZEN to HZEN was measured on the basis of the reduction in absorption at the wavelength of 315 nm. The molar extinction coefficients (ϵ) of ZEN and HZEN were determined experimentally and were found to amount to $0.0078895 \text{ L } \mu\text{mol}^{-1} \text{ cm}^{-1}$ and $0.0030857 \text{ L } \mu\text{mol}^{-1} \text{ cm}^{-1}$. The extinction coefficients have a strong dependence on pH and therefore the activity must always be measured precisely at the same pH and preferably also in the same matrix. The measurements were performed in a 50 mM Tris-HCl pH = 8.2 buffer solution in quartz cuvettes in a wavelength range of 200 to 2500 nm in a UV-VIS photometer (Hitachi U-2001) at 32°C.

A 2.08 mM ZEN solution (40 vol % ACN + 60 vol % H₂O) was used as the ZEN substrate stock solution. To prepare this solution, ZEN in crystalline form (Biopure Standard from Romer Labs, article no. 001109, purity at least 98 %) was weighed and dissolved accordingly. The ZEN substrate dilutions (0.79 μM , 1.57 μM , 2.36 μM , 3.14 μM , 4.71 μM , 6.28 μM , 7.85 μM , 9.42 μM , 10.99 μM , 12.56 μM , 14.13 μM , 15.71 μM , 17.28 μM and 18.85 μM) were prepared with 50 mM Tris-HCl pH = 8.2. The polypeptide solutions were diluted to a final concentration of approximately 70 ng/mL using 50 mM Tris-HCl buffer pH = 8.2. The ZEN substrate dilutions were preheated to 32°C in a water bath.

100 μL portions of the respective ZEN substrate dilution were mixed with 0.2 μL polypeptide solution, and the absorption was measured for 5 minutes, whereupon each combination of polypeptide solution and ZEN substrate dilution was measured at least twice.

Taking into account the extinction coefficients of ZEN and HZEN, the reaction rate was calculated for each substance concentration on the basis of the slope in the absorption over time.

The designations " K_M value" or "Michaelis-Menten constant" relate to a parameter for describing the enzymatic affinity of the units μM or mM, which are calculated with the help of the linear Hanes plots according to H. Bisswang (2002, Enzyme Kinetics, ISBN 3-527-30343-X, page 19), wherein the function "enzyme kinetics, single substrate" in the SigmaPlot 12.0 program is preferably used for this purpose. The designations "catalytic

constant of the enzyme reaction" or " k_{cat} value" relate to a parameter for describing the conversion rate of a polypeptide and/or enzyme, which is given in s^{-1} and is preferably calculated with the help of the "enzyme kinetic, single substrate" function of the SigmaPlot 12.0 program. The "maximum enzyme rate" or " v_{max} value" is given in units of $\mu M/s$ or
5 mM/s and is determined with the help of the linear Hanes plot by analogy with the K_M value, wherein the function "enzyme kinetic, single substrate" of the SigmaPlot 12.0 program is preferably used for this.

The specific activity was calculated by means of v_{max} and the enzyme concentration used
10 according to the equation

$$\text{Specific activity (U/mg)} = \frac{v_{max} (\mu M/s) \times 60 (s/min)}{\text{enzyme concentration (mg/L)}}$$

wherein one unit is defined as hydrolysis of 1 μmol ZEN per minute at 32°C in 50 mM Tris-HCl buffer solution, pH = 8.2.

15 The raw data for determination of the enzyme parameters K_M , v_{max} , k_{cat} and the specific activity are given below for the polypeptide having the sequence ID no. 1. Table 3 shows the reaction rates at the respective ZEN substrate concentrations, while Figure 2 shows the respective Michaelis-Menton graphs and Table 4 shows the corresponding enzyme kinetic parameters. The enzyme solution that was used had a concentration of 68 ng/L.

Table 3: Reaction rates of the polypeptide with sequence ID no. 1 at different ZEN concentrations.

ZEN substrate dilution (μM)	Measurement 1 reaction rate ($\mu\text{M/s}$)	Measurement 2 reaction rate ($\mu\text{M/s}$)
0.79	0.0073	0.0071
1.57	0.0087	0.0082
2.36	0.0095	0.0080
3.14	0.0101	0.0073
4.71	0.0103	0.0087
6.28	0.0096	0.0088
7.85	0.0084	0.0088
9.42	0.0111	0.0087
10.99	0.0093	0.0081
12.56	0.0100	0.0086
14.13	0.0089	0.0101
15.71	0.0089	0.0090
17.28	0.0100	0.0074
18.85	0.0100	0.0085

Table 4: Enzyme kinetics parameters of the polypeptide having sequence ID no. 1.

	v_{max} ($\mu\text{M/s}$)		K_{M} (μM)		k_{cat} (s^{-1})		Specific activity (U/mg)	
Measurement	1	2	1	2	1	2	1	2
Value	0.00993	0.008756	0.2172	0.1898	5.44	4.79	8.76	7.73
Average	0.009343		0.2035		5.12		8.25	

5

The specific activities of the polypeptides tested are 8.25 U/mg for sequence ID no. 1, 10.56 U/mg for sequence ID no. 2, 8.36 U/mg for sequence ID no. 3, 8.33 U/mg for sequence ID no. 4, 8.56 U/mg for sequence ID no. 5, 9.95 U/mg for sequence ID no. 6, 3.83 U/mg for sequence ID no. 7, 2.57 U/mg for sequence ID no. 8, 4.87 U/mg for sequence ID no. 9, 5.12 U/mg for sequence ID no. 10, 3.88 U/mg for sequence ID no. 11, 2.78 U/mg for sequence ID no. 12, 6.43 U/mg for sequence ID no. 13, 3.33 U/mg for sequence ID no. 14 and 7.76 U/mg for sequence ID no. 15.

10

The specific activities of the polypeptide variants tested are listed in Table 5 and Table 6.

Table 5: Specific activity of functional variants of the polypeptides having sequence ID no. 1; conserved amino acid sequence segments, in which the mutation(s) is/are located, and sequence identity of the functional variants for the parental sequence having sequence ID no. 1. The position of the mutations is given in relation to the amino acid sequence having sequence ID no. 1. The sequence identity was determined by means of BLAST, as described in Example 2.

n	Mutation(s)	Mutation(s) in range	Identity with SEQ ID No. 1	Specific activity (U/mg)
ZH1-A-001	N25D	A	99.7 %	8.10
ZH1-A-002	F27Y	A	99.7 %	7.93
ZH1-A-003	F27H	A	99.7 %	7.78
ZH1-A-004	R35K	A	99.7 %	8.98
ZH1-A-005	R35Q	A	99.7 %	8.56
ZH1-A-006	N25D/S29P/V42I/V43T	A	98.8 %	7.84
ZH1-A-007	I26V/R31A/F32Y/F46Y	A	98.8 %	8.61
ZH1-A-S02	N25D/I26V/F27Y/S29P/R31A/F32Y/ R35K/V37A/V42I/V43T/F46Y	A	96.6 %	8.73
ZH1-A-S03	N25D/I26V/F27H/S29P/R31A/F32Y/ R35Q/V42I/V43T/F46Y	A	97.0 %	8.52
ZH1-B-001	D53G	B	99.7 %	8.10
ZH1-B-002	N54M	B	99.7 %	8.41
ZH1-B-003	N54R	B	99.7 %	8.33
ZH1-B-004	S69G	B	99.7 %	8.06
ZH1-B-005	P72E	B	99.7 %	8.65
ZH1-B-006	P72R	B	99.7 %	8.78
ZH1-B-S02	N54M/L57V/L60I/S69G/P72E/V73A	B	98.2 %	8.51
ZH1-B-S03	D53G/N54R/L57V/L60I/P72E/V73A	B	98.2 %	8.56
ZH1-B-S04	N54R/L57V/L60I/P72E/V73A	B	98.5 %	8.96
ZH1-B-S14	N54R/L58V/L59P/L60V/T64G/P72R/ G75P/L77P	B	97.6 %	8.68

ZH1-C-001	N80H	C	99.7 %	8.24
ZH1-C-002	N80D	C	99.7 %	8.48
ZH1-C-003	F84Y	C	99.7 %	8.65
ZH1-C-S06	N80H/F84Y	C	99.4 %	8.88
ZH1-C-S10	N80H/F84H	C	99.4 %	8.32
ZH1-C-S14	E79R/N80D	C	99.4 %	8.45
ZH1-D-001	T95S	D	99.7 %	8.53
ZH1-D-002	R99K	D	99.7 %	8.25
ZH1-D-003	V123I	D	99.7 %	8.17
ZH1-D-004	A125G	D	99.7 %	8.36
ZH1-D-005	G126A	D	99.7 %	8.41
ZH1-D-006	G130A	D	99.7 %	8.69
ZH1-D-007	G130V	D	99.7 %	8.54
ZH1-D-008	G131A	D	99.7 %	8.71
ZH1-D-009	N127D	D	99.7 %	8.29
ZH1-D-010	N127Q	D	99.7 %	8.34
ZH1-D-011	A141S	D	99.7 %	8.67
ZH1-D-012	F106W	D	99.7 %	7.84
ZH1-D-013	I118V	D	99.7 %	8.37
ZH1-D-014	I118V/V123L	D	99.4 %	8.55
ZH1-D-015	I118V/K119R/L132V	D	99.1 %	8.86
ZH1-D-016	W96Q/F106W/L116G/V122A	D	98.8 %	8.65
ZH1-D-017	Q91R/N105D/K119G/A141S/M142K	D	98.5 %	8.46
ZH1-D-S02	T95S/T97A/R99K/I118V/V123I/L132V/A141S	D	97.7 %	8.66
ZH1-D-S03	T95S/R99K/I118V/K119R/L132V/A141S	D	98.2 %	9.32
ZH1-D-S04	T95S/R99K/I118V/L132V/A141S	D	98.5 %	9.15
ZH1-D-S05	T95S/R99K/I114M/I118V/K119R/L132V/A141S	D	97.7 %	8.84

ZH1-D-S07	R99G/A115D/K119G/P121T/V123I/A125S/L132V/L133V/S138A/Y140F/A141S/M142L	D	96.3 %	8.79
ZH1-D-S08	R93K/W96Q/R99G/D104N/N105L/F106M/A115S/V123I/A125S/G144N	D	97.0 %	8.86
ZH1-D-S09	R99G/S102N/D104N/N105T/F106W/L110V/V111E/A115D/K119G/V122T/V123L/L132V/L133I/S138A/M142K	D	95.4 %	8.99
ZH1-D-S10	W96R/S102T/F106I/I114L/A115S/L116G/K119G/V122A/V123F/A125S/A134S/Y140F/M142E	D	96.0 %	9.12
ZH1-D-S11	W96R/R99G/S102T/F106V/I114L/A115D/L116G/K119G/V122A/V123F/A125S/N127L/L133A/A134S/Y140F/M142K	D	95.1 %	8.54
ZH1-D-S12	S94T/R99G/S102T/N105I/L110V/A115D/K119G/P121E/V122T/V123L/V124I/L133I/A134G/S138A/Y140F/M142K	D	95.1 %	8.69
ZH1-D-S13	R93Q/R99G/N105T/R112K/A115D/L116I/A125S/N127L/L132V/L133V/A134S/Y140F/M142K	D	96.0 %	8.47
ZH1-D-S14	Q91R/W96R/N105D/I114L/I118V/K119R/V122A/L132V/L137S	D	97.3 %	8.55
ZH1-E-001	Y165C	E	99.7 %	8.46
ZH1-E-002	Y165H	E	99.7 %	8.33
ZH1-E-003	P163T	E	99.7 %	7.95
ZH1-E-004	A154P/Y165C	E	99.4 %	8.13
ZH1-E-S02	P163T/A164T/Y165C/V169I/L170R	E	98.5 %	8.83
ZH1-E-S05	A154P/Y165H/L170R	E	99.1 %	9.65
ZH1-F-001	Y180F	F	99.7 %	8.35
ZH1-F-002	D182T	F	99.7 %	8.41

ZH1-F-003	D182K	F	99.7 %	8.19
ZH1-F-004	Y180F/R181V/I190V	F	99.1 %	8.56
ZH1-F-S04	Y180F/D182T/F183Y/I190V/G191S	F	98.5 %	8.56
ZH1-F-S06	Y180F/D182T/F183Y/I190V	F	98.8 %	8.64
ZH1-F-S10	E178A/R181V/D182K/F183Y	F	98.8 %	7.55
ZH1-H-001	T236K	H	99.7 %	8.09
ZH1-H-002	V237F	H	99.7 %	8.11
ZH1-H-003	E234G	H	99.7 %	8.54
ZH1-H-S02	F233W	H	99.7 %	8.37
ZH1-H-S03	F233Y	H	99.7 %	8.64
ZH1-H-S04	F233H	H	99.7 %	8.36
ZH1-H-S06	A231V/F233Y	H	99.4 %	8.54
ZH1-H-S09	F232W/F233A/E234T/G235D/L239A	H	98.5 %	8.83
ZH1-I-001	H240N	I	99.7 %	8.54
ZH1-I-002	H240S	I	99.7 %	8.79
ZH1-I-003	D244E/R245Y	I	99.4 %	8.42
ZH1-I-S02	D244E/R245Q/M246L	I	99.1 %	8.36
ZH1-I-S03	H240N/D244E	I	99.4 %	9.26
ZH1-I-S06	H240S/D244E	I	99.4 %	9.02
ZH1-I-S07	L239Q/H240T/R245Y	I	99.1 %	8.41
ZH1-J-001	Q249R	J	99.7 %	8.36
ZH1-J-002	T252V	J	99.7 %	7.94
ZH1-J-S02	I254V	J	99.7 %	8.55
ZH1-J-S03	Q249R/K251N/I254V	J	99.1 %	9.03
ZH1-J-S07	T252V/I254M	J	99.4 %	7.81
ZH1-J-S10	T252V/I254V	J	99.4 %	7.97
ZH1-K-S05	A260M	K	99.7 %	8.64
ZH1-K-S11	A260F	K	99.7 %	8.82
ZH1-K-S13	A260S	K	99.7 %	9.01
ZH1-L-001	E266Y	L	99.7 %	8.46

ZH1-L-002	E266D	L	99.7 %	8.31
ZH1-L-003	T262G	L	99.7 %	8.32
ZH1-L-004	T262D/E266D/	L	99.4 %	8.56
ZH1-L-005	T262G/I263T/	L	99.4 %	8.68
ZH1-L-S02	E266D/E269H	L	99.4 %	8.59
ZH1-L-S04	I263T/E269N	L	99.4 %	8.73
ZH1-L-S06	E269N	L	99.7 %	8.69
ZH1-L-S13	E266Y/E269N	L	99.4 %	8.33
ZH1-M-001	L274M	M	99.7 %	8.29
ZH1-M-002	L274C	M	99.7 %	8.37
ZH1-M-S02	L277E	M	99.7 %	8.96
ZH1-M-S07	L274M/A279V	M	99.4 %	8.23
ZH1-M-S08	L274T/L277F	M	99.4 %	8.63
ZH1-M-S11	L274C/L277I	M	99.4 %	8.51
ZH1-N-001	H297L	N	99.7 %	8.27
ZH1-N-002	H298V/L302S	N	99.4 %	9.03
ZH1-N-S02	H298V	N	99.7 %	8.94
ZH1-N-S09	H298L/P299D	N	99.4 %	8.37
ZH1-O-001	L307Q	O	99.7 %	8.62
ZH1-O-002	F308S	O	99.7 %	8.57
ZH1-O-S02	L307Q/A311P	O	99.4 %	8.34
ZH1-O-S03	L307Q/F308S	O	99.4 %	8.74
ZH1-O-S06	L307Q/F308S/D309A	O	99.1 %	9.18
ZH1-B/H-001	D53G/N54R/L57V/L60I/P72E/V73A/ F233V/E234G/V237F	B+H	97.3 %	9.26
ZH1-C/D-001	N80H/F84Y/T95S/R99K/I118V/K119 R/L132V/A141S	C+D	97.6 %	9.31
ZH1-D/K-001	T95S/T97A/R99K/I118V/V123I/L132 V/A141S/A260M	D+K	97.6 %	9.66
ZH1-D/M-001	T95S/T97A/R99K/I118V/V123I/L132 V/A141S/L277E	D+M	97.6 %	10.63

ZH1-K/N-001	A260M/H298V	K+N	99.4 %	8.94
ZH1-K/L-001	A260M/T262D/E266D/E269H	K+L	98.8 %	9.03
ZH1-K/L-002	A260M/T262G/I263T/E269N	K+L	98.8 %	8.84
ZH1-N/O-001	Q296A/H298V/L307Q/A311P	N+O	98.8 %	9.26
ZH1-N/O-002	Q296E/H298V/L302S/L307Q/F308S	N+O	98.5 %	9.46
ZH1-C/D/J-001	N80H/F84Y/T95S/R99K/I118V/L132V/A141S/Q249R/K251N/I254V	C+D+J	97.0 %	9.97
ZH1-B/D/K-001	D53G/N54R/L57V/L60I/P72E/V73A/T95S/R99K/I114M/I118V/K119R/L132V/A141S/A260M	B+D+K	95.7 %	10.78
ZH-J/K/L-001	I254V/I256L/A260M/T262G/I263T/E269N	J+K+L	98.2 %	9.11
ZH1-J/K/LM-001	I254V/I256L/A260M/T262D/E266D/E269H/L271V	J+K+L+M	97.7 %	9.14
ZH1-B/C/D/J-002	E79R/N80D/D53G/N54R/L57V/L60I/P72E/V73A/W96R/R99G/S102T/F106V/I114L/A115D/L116G/K119G/V122A/V123F/A125S/N127L/L133A/A134S/Y140F/M142K/T252V/I254V	B+C+D+J	92.1 %	11.31
ZH1-DEL-001	ΔP212	-	99.7 %	8.56
ZH1-DEL-002	ΔG5/ΔT6/ΔR7/ΔS8/ΔE9/ΔA10/ΔA11/ΔD12/ΔA13/ΔA14/ΔT15/ΔQ16/ΔA17/ΔR18/ΔQ19	-	95.4 %	8.37
ZH1-DEL-003	ΔN327/ΔD328	-	99.4 %	8.27
ZH1-A/B/C-001	N25D/I26V/F27Y/S29P/R31A/F32Y/R35K/V37A/V42I/V43T/F46Y/N54R/	A+B+C	89.6 %	9.54

	L58V/L59P/L60V/T64G/P72R/G75P/L77P/R99G/S102N/D104N/N105T/F106W/L110V/V111E/A115D/K119G/V122T/V123L/L132V/L133I/S138A/M142K			
ZH1-DEL/B/C/D/J-001	ΔG5/ΔT6/ΔR7/ΔS8/ΔE9/ΔA10/ΔA11/ΔD12/ΔA13/ΔA14/ΔT15/ΔQ16/ΔA17/ΔR18/ΔQ19/ΔP212/ΔN327/ΔD328/E79R/N80D/D53G/N54R/L57V/L60I/P72E/V73A/W96R/R99G/S102T/F106V/I114L/A115D/L116G/K119G/V122A/V123F/A125S/N127L/L133A/A134S/Y140F/M142K/T252V/I254V	B+C+D+J	86.6 %	11.52
ZH1-DEL/A/B/C/D/J-001	ΔG5/ΔT6/ΔR7/ΔS8/ΔE9/ΔA10/ΔA11/ΔD12/ΔA13/ΔA14/ΔT15/ΔQ16/ΔA17/ΔR18/ΔQ19/ΔP212/ΔN327/ΔD328/N25D/I26V/F27Y/S29P/R31A/F32Y/R35K/V37A/V42I/V43T/F46Y/E79R/N80D/D53G/N54R/L57V/L60I/P72E/V73A/W96R/R99G/S102T/F106V/I114L/A115D/L116G/K119G/V122A/V123F/A125S/N127L/L133A/A134S/Y140F/M142K/T252V/I254V	A+B+C+D+J	83.3 %	10.92
ZH1-001	L302S	-	99.7 %	8.31

Table 6: Specific activities of functional variants of the polypeptide having sequence ID no. 2. The position of the mutation(s) is relative to the amino acid sequence with sequence ID no. 2. The sequence identity was determined by means of BLAST as described in example 2.

Variant	Mutation(s)	Identity with SEQ ID No. 2	Specific activity (U/mg)
ZH2-001	D3D(GTRSEAADAATQARQL)	93.6 %	10.15
ZH2-002	D8N/V9I/Y10F	99.0 %	10.42

ZH2-003	M37N/E55P/A56V/V101I/S124A/F194 FP/T146P/T147A/C148Y	97.0 %	10.58
ZH2-004	S187P/S188A/P189K/M190A/A191M/ R192Q/Y193L	97.7 %	10.43
ZH2-005	A262E/R263H/R265Q/L266D/L267I/M 268I/E269R	97.7 %	10.68
ZH2-006	D3D(GTRSEAADAATQARQL)/M37N/ E55P/A56V/V101I/S124A/F194FP/T14 6P/T147A/C148Y/S187P/S188A/P189 K/M190A/A191M/R192Q/Y193L/A262 E/R263H/R265Q/L266D/L267I/M268I/ E269R	86.1 %	10.71

Example 6: Degradation of ZEN and ZEN derivatives in contaminated corn

To determine the capabilities of polypeptides to degrade naturally occurring ZEN and ZEN
 5 derivatives in a complex matrix and at a low pH, contaminated corn was mixed with
 different concentrations of one of the polypeptides having the sequence ID numbers 1 to 6
 and the degradation of ZEN and ZEN derivatives was tracked.

The contaminated corn was ground and used in the degradation experiment wherein a
 10 batch would consist of 1 g ground contaminated corn, 8.9 mL 100 mM acetate buffer
 pH 4.0 and 0.1 mL polypeptide solution. Enriched and purified polypeptide solutions were
 prepared as described in example 5, diluting them to a concentration of 10 mU/mL,
 100 mU/mL and/or 1000 mU/mL. Thus in absolute amounts 1 mU (= 1 mU per gram corn),
 10 mU (= 10 mU per gram corn) and/or 100 mU (= 100 mU per gram of corn) were used
 15 in the batch. Each degradation batch was carried out in 25 mL and incubated at 37°C and
 100 rpm with agitation. Before adding the enzyme and/or after 1 hour of incubation, a
 sample of 1 mL was taken, the polypeptide was heat inactivated at 99°C for 10 minutes
 and the sample was stored at -20°C. After thawing the sample, the insoluble constituents
 were separated by centrifugation. Concentrations of ZEN and ZEN derivatives were
 20 measured by means of LC/MS/MS as described by M. Sulyok et al. (2007, Anal. Bioanal.
 Chem., 289, 1505-1523). The ZEN and ZEN derivative content in this corn was 238 ppb
 for ZEN, 15 ppb for α -ZEL, 23 ppb for β -ZEL, 32 ppb for Z14G and 81 ppb for Z14S. Table

7 shows the percentage reduction in the ZEN and ZEN derivative content in the degradation experiment.

5 Table 7: Reduction in ZEN and ZEN derivatives in percentage based on the starting content in the degradation experiment with different polypeptides and amounts of polypeptides.

Polypeptide	Amount in the batch	ZEN	α -ZEL	β -ZEL	Z14G	Z14S
SEQ ID No. 1	0.1 mU	83 %	≥ 80 %	70 %	78 %	80 %
	1 mU	96 %	≥ 80 %	76 %	≥ 80 %	92 %
	10 mU	97 %	≥ 80 %	≥ 85 %	≥ 80 %	94 %
SEQ ID No. 2	0.1 mU	87 %	≥ 80 %	73 %	≥ 80 %	84 %
	1 mU	97 %	≥ 80 %	78 %	≥ 80 %	90 %
	10 mU	99 %	≥ 80 %	≥ 85 %	≥ 80 %	96 %
SEQ ID No. 3	0.1 mU	79 %	79 %	67 %	73 %	75 %
	1 mU	85 %	≥ 80 %	72 %	79 %	82 %
	10 mU	92 %	≥ 80 %	78 %	≥ 80 %	88 %
SEQ ID No. 4	0.1 mU	82 %	78 %	65 %	76 %	80 %
	1 mU	89 %	≥ 80 %	73 %	≥ 80 %	86 %
	10 mU	93 %	≥ 80 %	82 %	≥ 80 %	91 %
SEQ ID No. 5	0.1 mU	79 %	76 %	66 %	78 %	80 %
	1 mU	83 %	≥ 80 %	73 %	≥ 80 %	81 %
	10 mU	91 %	≥ 80 %	79 %	≥ 80 %	86 %
SEQ ID No. 6	0.1 mU	93 %	≥ 80 %	75 %	≥ 80 %	90 %
	1 mU	95 %	≥ 80 %	82 %	≥ 80 %	92 %
	10 mU	98 %	≥ 80 %	≥ 85 %	≥ 80 %	96 %

Example 7: Additives containing polypeptide for hydrolytic cleavage of ZEN and/or ZEN derivatives

To prepare additives for hydrolytic cleavage of ZEN, fermentation supernatants of polypeptides expressed by *P. pastoris* and having the sequence ID numbers 1, 2, 6 and 13 were purified by microfiltration and ultrafiltration (exclusion limit: 10 kDa) under standard conditions and concentrated up to a dry substance concentration of approximately 9 % by weight. Following that, these polypeptide-containing solutions were also processed further to form dry powders under standard conditions in a spray dryer (Mini B290 from Büchi). These four powders were subsequently designated as Z1, Z2, Z6 and Z13. Z1, Z2, Z6 and/or Z13 were additionally mixed with bentonite having an average grain size of approximately 1 µm in a ratio of 1 % by weight of additives Z1, Z2, Z6 and/or Z13 and 99 % by weight bentonite in an overhead agitator. The resulting additives are designated as additives Z1.B, Z2.B, Z6.B and Z13.B. In addition, Z1, Z2, Z6 and Z13 were mixed with bentonite and a vitamin trace element concentrate in a ratio of 0.1 % by weight additive Z1, Z2, Z6 and/or Z13, 0.9 % by weight vitamin trace elements concentrate and 99 % by weight bentonite in an overhead agitator. The resulting additives were designated as additive Z1.BVS, Z2.BVS, Z6.BVS and Z13.BVS. 100 g of the additives Z1.BVS, Z2.BVS, Z6.BVS and Z13.BVS contained 200 mg iron sulfate, 50 mg copper sulfate, 130 mg zinc oxide, 130 mg manganese oxide, 2.55 mg calcium carbonate, 160 mg vitamin E, 6.5 mg vitamin K3, 6.5 mg vitamin B1, 14 mg vitamin B2, 15 mg vitamin B6, 0.15 mg vitamin B12, 150 mg nicotinic acid, 30 mg pantothenic acid and 5.3 mg folic acid.

The additives were extracted for 30 minutes in a 50 mM Tris-HCl buffer pH = 8.2 and diluted further in the same buffer so that the final concentration of polypeptide was approximately 70 ng/mL.

Following that, the zearalenone-degrading effect of these solutions was determined as described in Example 5. The corresponding activities were 8.230 U/g for Z1, 9.310 U/g for Z2, 9.214 U/g for Z6, 83 U/g for Z1.B, 92 U/g for Z2.B, 90 U/g for Z2.C, 57 U/g for Z13.B, 8 U/g for Z1.BVS, 9 U/g for Z2.BVS, 9 U/g for Z6.BVS and 6 U/g for Z13.BVS.

The ability to degrade ZEN derivatives α -ZEL, β -ZEL, α -ZAL, β -ZAL, Z14G, Z14S and ZAN by the additives Z1, Z2, Z6, Z13, Z1.B, Z2.B, Z6.B, Z13.B, Z1.BVS, Z2.BVS, Z6.BVS and Z13.BVS was tested as described in Example 4, but instead of 100 µL of a cell lysate, 100 µL of a polypeptide solution with a polypeptide concentration of approximately 70

ng/mL was used. After incubating for 6 hours, only max. 15 % of the starting amount was present as unhydrolyzed ZEN derivative.

Example 8: Optimum temperature

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To determine the temperature optimum of the polypeptides having SEQ ID numbers 1, 2, 5, 6, 7, 9, 11, 12 and 15, they were cloned with a C-terminal 6xHis tag as described in example 1, expressed in *E. coli* and purified. In preliminary experiments, the concentration at which a complete conversion of ZEN could be ensured under the experimental conditions was determined (Teorell-Stenhagen buffer (Teorell and Stenhagen, A universal buffer for the pH range of 2.0 to 12.0. Biochem Ztschrft, 1938, 299:416-419), pH 7.5 with 0.1 mg/mL BSA at 30°C) after an experimental time of 3 hours. The preparations were used in the concentrations thus determined in the degradation batches for determining the optimum temperature. The experiments were carried out in a PCR Cycler (Eppendorf) using the temperature gradient function at 20°C ± 10°C, at 40°C ± 10°C and, if necessary, at 60°C ± 10°C (10 temperatures in the respective range; temperatures predefined by the PCR cycler). For the batches Teorell-Stenhagen buffer was mixed with the corresponding enzyme concentration and 0.1 mg/mL BSA plus 5 ppm ZEN at the respective optimum pH. Batches with 0.1 mg/mL BSA and 5 ppm ZEN without addition of an enzyme were used as negative controls. After 0 h, 0.5 h, 1 h, 2 h and 3 h incubation time, a sample was taken per incubation temperature, heat inactivated for 10 minutes at 99°C and stored at -20°C. After thawing, the samples were transferred to HPLC vials. ZEN, HZEN and DHZEN were analyzed by HPLC-DAD. To do so the metabolites were separated chromatographically on a Zorbax SB-Aq C18 column with the dimensions 4.6 mm × 150 mm and a particle size of 5 µm. A methanol-water mixture with 5 mM ammonium acetate was used as the mobile phase. The UV signal at 274 nm was recorded. The metabolites were quantified by including entrained standard series. The optimum temperatures were determined on the basis of the slopes determined for the degradation curves, where the optimum temperature was defined as the temperature at which the slope was the greatest. Table 8 shows the optimum temperatures.

Table 8: Optimum temperatures of the polypeptides.

SEQ ID No. 1	SEQ ID No. 2	SEQ ID No. 5	SEQ ID No. 6	SEQ ID No. 7	SEQ ID No. 9	SEQ ID No. 11	SEQ ID No. 12	SEQ ID No. 15
38°C	41°C	50°C	51°C	31°C	35°C	50°C	26°C	41°C

Example 9: Thermal stability

To determine the thermal stability of polypeptides with the SEQ ID numbers 1, 2, 5, 6, 7, 9, 11, 12 and 15, they were cloned with a C-terminal 6xHis tag as described in Example 1, expressed in *E. coli* and purified. They were then incubated in the PCR cyclor with a gradient function at the respective optimum temperature $\pm 10^{\circ}\text{C}$. After 0 min, 15 min, 30 min and 60 min, one sample was taken per batch and per temperature. These pre-incubated samples were then used in a degradation experiment in the Teorell-Stenhagen buffer at the respective optimum pH with 0.1 mg/mL BSA and 5 ppm ZEN. In preliminary experiments, the concentration at which a complete reaction of ZEN could be ensured after an experimental duration of 3 hours under the experimental conditions (Teorell-Stenhagen buffer, pH 7.5 with 0.1 mg/mL BSA at 30°C) was determined for each polypeptide. The respective enzyme concentration thereby determined was used in the batches. The degradation batches were incubated at 30°C . Sampling was performed after 0 h, 0.5 h, 1 h, 2 h and 3 h incubation time. Next, the polypeptides were heat-inactivated for 10 minutes at 99°C and the samples were stored at -20°C . After thawing the samples were transferred to HPLC vials and analyzed by HPLC-DAD, as described in Example 8.

Thermal stability is defined as the temperature at which the polypeptides have a 50 % residual activity in comparison with the optimum temperature after 15 minutes of pre-incubation. As a measure of the activity, the slope in the degradation curves is used. The temperature stabilities are shown in Table 9.

Table 9: Temperature stability of the polypeptides (50 % residual activity after pre-incubation for 15 minutes).

SEQ ID	SEQ ID	SEQ ID	SEQ ID	SEQ ID	SEQ ID	SEQ ID	SEQ ID	SEQ ID
No. 1	No. 2	No. 5	No. 6	No. 7	No. 9	No. 11	No. 12	No. 15
38°C	34°C	54°C	61°C	28°C	44°C	55°C	40°C	49°C

Example 10: Optimum pH

To determine the optimum pH of the polypeptides having the SEQ ID numbers 1, 2, 5, 6, 7, 9, 11, 12 and 15, they were cloned with a C-terminal 6xHis tag as described in Example 1, expressed in *E. coli* and purified. In preliminary experiments, the concentration at which a complete conversion of ZEN could be ensured after an experimental duration of 3 hours

under the experimental conditions was determined for each polypeptide (Teorell-Stenhagen buffer, pH 7.5 with 0.1 mg/mL BSA at 30°C). The respective enzyme concentration was used in the batches. The degradation batches were carried out in Stenhagen buffer at pH levels of 3.0, 4.0, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 11.0 and 12.0. For the degradation batches with 0.1 mg/mL BSA and 5 ppm ZEN, incubation was done at 30°C. Batches in Teorell-Stenhagen buffer were used as the negative controls at pH 3.0, pH 7.0 and pH 12.0 with 0.1 mg/mL BSA and 5 ppm ZEN. Sampling was performed after an incubation time of 0 h, 0.5 h, 1 h, 2 h and 3 h. Next the polypeptides were heat-inactivated for 10 minutes at 99°C and the samples were stored at -20°C. After thawing, the samples were transferred to HPLC vials and analyzed by HPLC-DAD as described in Example 8. The optimum pH was determined on the basis of the slopes found for the degradation curves, wherein the pH at which the slope was the greatest was defined as the optimum pH. Table 10 shows the optimum pH levels.

Table 10: Optimum pH of the polypeptides.

SEQ ID No. 1	SEQ ID No. 2	SEQ ID No. 5	SEQ ID No. 6	SEQ ID No. 7	SEQ ID No. 9	SEQ ID No. 11	SEQ ID No. 12	SEQ ID No. 15
8.2	8.5	7.0-8.0	7.0-7.5	7.5-8.5	7.0-7.5	8.0	7.0-7.5	7.5

Example 11: pH stability at pH 5.0

To determine the pH stability, the polypeptides from Example 10 were incubated for one hour at 25°C in Teorell-Stenhagen buffer at pH 5.0 and at the respective optimum pH. These pre-incubated samples were used in a degradation experiment in the same concentrations of the respective polypeptide as those used to determine the optimum pH in 100 mM Tris-HCl buffer at the respective optimum pH with 0.1 mg/mL BSA and 5 pm ZEN in the batch. The batches were incubated at the respective optimum temperature. Sampling was performed after 0 h, 0.5 h, 1 h, 2 h and 3 h incubation time. Next the polypeptides were heat inactivated for 10 minutes at 99°C and the samples were stored at -20°C. After thawing, the samples were transferred to HPLC vials and analyzed by means of HPLC-DAD as described in Example 8. The pH stability is defined as the percentage residual activity of the polypeptides at pH 5.0 relative to the activity at the respective optimum pH. The pH stabilities for 5.0 are shown in Table 11.

Table 11: pH stability of the polypeptides at pH 5.0.

SEQ ID No. 1	SEQ ID No. 2	SEQ ID No. 5	SEQ ID No. 6	SEQ ID No. 7	SEQ ID No. 9	SEQ ID No. 11	SEQ ID No. 12	SEQ ID No. 15
3 %	17 %	79 %	80 %	100 %	22 %	87 %	98 %	19 %

Example 12: ZEN degradation experiment

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The degradation of ZEN to HZEN and DHZEN was performed as an example for the polypeptides with sequence ID numbers 1, 2, 5, 6, 7, 9, 11, 12 and 15. The degradation batches were carried in Teorell-Stenhagen buffer pH 7.5 with 0.1 mg/mL BSA and 5 ppm ZEN. The degradation batches were incubated at 30°C. Sampling was performed after 0 h, 0.5 h, 1 h, 2 h and 3 h incubation time. Next the polypeptides were heat-inactivated for 10 minutes at 99°C and the samples were stored at -20°C. After thawing, the samples were transferred to HPLC vials and analyzed by HPLC-DAD, as described in Example 8. The polypeptide concentration was selected so that complete degradation was achieved after approximately 3 hours. Figure 3 shows the degradation kinetics, where the y axis shows the concentration of ZEN, HZEN and DHZEN in micromoles per liter ($\mu\text{mol/L}$) and the x axis shows the incubation time in hours (h).

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* μM denotes micromolar and corresponds to the unit $\mu\text{mol/L}$

Patent Claims

1. A polypeptide that hydrolytically cleaves zearalenone and/or at least one zearalenone derivative selected from the group consisting of α -zearalenol and β -zearalenol, characterized in that the polypeptide is a hydrolase with an amino acid sequence ID No. 1 or a functional variant thereof, wherein a sequence identity of at least 84 % exists between the functional variants and the amino acid sequence ID No. 1, and wherein the functional variants hydrolyze zearalenone and/or at least one zearalenone derivative.
2. The polypeptide according to claim 1, characterized in that the polypeptide has at least one mutation of the amino acid sequence with respect to sequence ID No. 1 in at least one of the following positions selected from the group: 22, 23, 25, 26, 27, 29, 31, 32, 35, 37, 42, 43, 46, 51, 53, 54, 57, 60, 69, 72, 73, 78, 80, 84, 88, 95, 97, 99, 114, 118, 119, 123, 132, 141, 146, 148, 149, 154, 163, 164, 165, 169, 170, 172, 176, 180, 182, 183, 190, 191, 194, 196, 197, 198, 201, 204, 205, 206, 207, 208, 209, 210, 212, 213, 214, 216, 217, 220, 221, 222, 229, 231, 233, 238, 240, 244, 245, 246, 248, 249, 251, 254, 256, 260, 262, 263, 266, 269, 271, 277, 280, 281, 282, 283, 284, 285, 286, 287, 292, 296, 298, 302, 307, 308, 309, 311, 314, 317, 319, 321, 323, 325 and 326.
3. The polypeptide according to claim 1 or 2, characterized in that it has at least one mutation of the amino acid sequence with respect to sequence ID No. 1 selected from the group D22A, S23Q, S23L, N25D, I26V, F27Y, F27H, S29P, R31A, F32Y, R35K, R35Q, V37A, V42I, V43T, F46Y, S51E, S51D, D53G, N54M, N54R, L57V, L60I, S69G, P72E, V73A, A78S, N80H, F84Y, I88L, T95S, T97A, R99K, I114M, I118V, K119R, V123I, L132V, A141S, I146V, I146L, A148G, A149V, A154P, P163T, A164T, Y165C, Y165H, V169I, L170R, A172G, A176M, A176V, Y180F, D182T, F183Y, I190V, G191S, K194T, K194E, F196Y, V197C, V197R, E198R, E198S, K201D, K201G, P204S, P204A, A205S, K206P, A207M, M208A, Q209R, L210A, L210S, Δ P212, T213V, P214A, E216T, E216G, A217I, N220H, L221M, K222R, K222Q, G229A, A231V, F233W, F233Y, F233H, A238G, H240N, H240S, D244E, R245Q, M246L, S248T, S248N, S248G, Q249R, K251N, I254V, I256L, A260M, T262D, T262G, I263T, E266D, E269H, E269N, L271V, L277E, E280A, E280L, H281R, H281Q, A282V, Q283R, D284L, D284R, I285L, I286M, R287E, R287D, R292K, R292T, Q296A, Q296E, H298V, L302S, L307Q, F308S, D309A, A311P, A314V, L317F, S319Q, S319P, S319R, S321A, S321T, T323A, P325A, A326P.

4. The polypeptide according to any one of claims 1 to 3, characterized in that it contains at least one of the following amino acid motifs selected from the group of sequence ID numbers 32 to 69.

5 5. An additive that hydrolytically cleaves zearalenone and/or at least one zearalenone derivative to yield feed products for pigs, poultry or aquaculture, for addition to foodstuffs or to distillers dried grain and solubles, characterized in that the additive contains at least one polypeptide having an amino acid sequence ID No. 1 or a functional variant thereof, wherein a sequence identity between the functional variant and the amino acid sequence
10 amounts to at least 84 % and auxiliary substances are also present.

6. The additive according to claim 5, characterized in that at least one polypeptide according to any one of the claims 1 to 4 is contained therein.

15 7. The additive according to claim 5 or 6, characterized in that the auxiliary substances are selected from at least one inert carrier as well as optional additional ingredients, such as vitamins and/or minerals and/or enzymes and/or other components for detoxifying mycotoxins.

20 8. The additive according to claim 5, 6 or 7, characterized in that at least one polypeptide according to any one of claims 1 to 5 is present in the additive in a concentration of at most 10,000 U/g, preferably at most 1000 U/g, more preferably at most 100 U/g and most preferably at most 10 U/g.

25 9. The additive according to any one of claims 5 to 8, characterized in that the additive is present in encapsulated or coated form.

10. A use of at least one polypeptide having an amino acid sequence ID No. 1 or a functional variant thereof, wherein additives containing a sequence identity between the functional variant and the amino acid sequence amounts to at least 84 %, for hydrolytic
30 cleavage of zearalenone and/or at least one zearalenone derivative in animal feed products, in particular pigs, poultry and aquaculture, in food items or in distillers dried grain and solubles.

35 11. The method for hydrolytic cleavage of zearalenone and/or at least one zearalenone derivative, characterized in that the zearalenone and/or at least one

zearalenone derivative is/are hydrolyzed by a polypeptide having an amino acid sequence ID No. 1 or a functional variant thereof, wherein the sequence identity between the functional variant and the amino acid sequence is at least 84 %.

5 12. The method according to claim 11, characterized in that the polypeptide is used in an additive according to any one of claims 6 to 10.

10 13. The method according to claim 12, characterized in that the polypeptide or the additive is mixed with a foodstuff or animal feed product contaminated with zearalenone and/or with at least one zearalenone derivative; the contaminated foodstuff or animal feed product is brought in contact with moisture, and the polypeptide or the additive hydrolyzes the zearalenone and/or at least one zearalenone derivative present in contaminated foodstuff or animal feed product.