Infinite Enzymes, Inc.; Jonesboro, AR

EARLY FOOD SAFETY EVALUATION FOR A NEW NON-PESTICIDAL PROTEIN PRODUCED BY A NEW PLANT VARIETY (Manganese-dependent peroxidase expressed in bioengineered corn (*Zea mays*, L.))

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This submission contains no Confidential Business Information

Table of Contents

Introduction

Infinite Enzymes, Inc. (IE) is submitting this document to US FDA/ Office of Food Additive Safety/ CFSAN as described in its early food safety evaluation guidance to be considered as a New Protein Consultation (NPC). IE has been growing bioengineered corn (maize) lines producing manganese-dependent peroxidase (MnP) for many years under USDA-APHIS permits and intends to scale up production to increase sales of its MnP products for a variety of potential environmental detoxification/bioremediation uses. The manganese-dependent peroxidase of note here has never been produced in large enough quantities or at low enough cost for IE's intended applications. While IE does not have current plans for using its MnP in food or feed products, this submission is intended to inform FDA of the presence of its MnP corn lines and demonstrate the food/feed safety of these lines in the unlikely event that its MnP-producing corn were to inadvertently enter the food or feed supply chains.

Characteristics of manganese-dependent peroxidase (EC 1.11.1.13)

The basic reaction of manganese-dependent peroxidase is shown below. Adding hydrogen peroxide begins the reaction by accepting the 2 electrons from the Fe center of the enzyme. The Fe core accepts 2 electrons from chelated Mn++ creating 2 Mn+++ ions that are thus activated to remove electrons from other molecules—the work of the enzyme completed. MnP may also have applications for industrial wastewater treatment (Xu, et al, 2017).

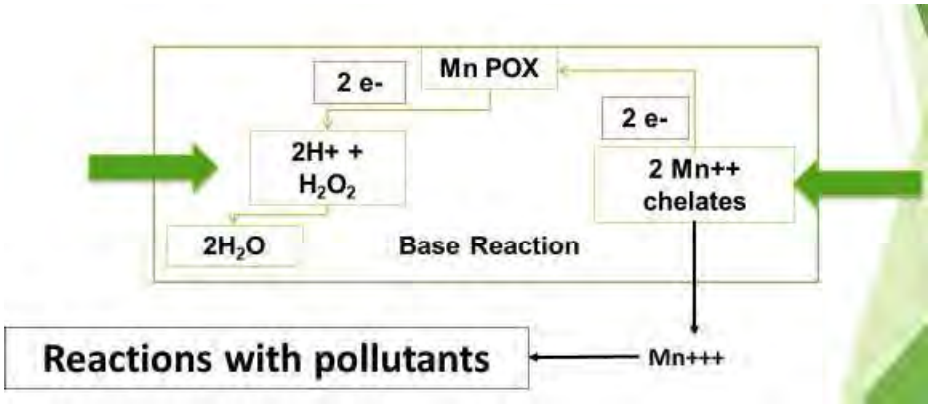

Figure 2. The basic manganese peroxidase (MnP) reaction scheme: MnP catalysis occurs in a series of oxidation-reduction reactions. In step one, hydrogen peroxide (or an organic peroxide) enters the active site of the enzyme. The oxygen binds to the iron core, then two electrons are transferred from Fe3+ to peroxide, breaking the bond to form water and a radical. The Fe3+ core then binds to one Mn2+, which donates an electron to the iron core, generating Mn3+, followed by a second electron extraction from a second Mn2+ to form the second Mn3+, regenerating the

ground state of the enzyme. The activated unstable Mn3+ can then extract electrons from secondary compounds (modified from Wikipedia: Manganese peroxidase - Wikipedia).

Maize contains numerous peroxidases of its own. A white paper describing those was submitted to APHIS-BRS as part of early IE permit applications and is included in this document in Appendix I.

Description of intended use/effect

Infinite Enzymes intends to grow its corn line(s) to provide scale-up so that it may extract manganese-dependent peroxidase to be used in environmental detoxification/ bioremediation efforts.

Corn as a plant and crop

Zea mays, L., is a food, feed, fiber, and fuel crop plant. It is grown on approximately 85-95 million acres in the U.S. every year. Roughly forty percent of the crop goes to ethanol production as a gasoline additive. Another 30-40% goes to animal feed for cattle, hogs, and poultry. A small percentage is exported, and small amounts are used in human food products. As corn has hundreds of uses in food, feed, and industrial products, USDA tracks its growing, exports, and imports closely through numerous programs and agencies (e.g., ERS, NASS, FAS, etc).

Corn is an open pollinated crop with separate male and female flowers. It is most often produced as a hybrid, and thus the male and female crosses to produce hybrids must be controlled. This is fortunate for the IE team in that when doing back crosses to generate inbred elite lines for the hybrid, selection for higher expression of the transgene can be accomplished over the several generations required for breeding.

Identity of the manganese-dependent peroxidase source gene

The manganese peroxidase gene (MnP) inserted into this corn is for the major (first) isozyme from *Phanerochaete chrysosporium*[1], a white rot fungus. Transformation was achieved using the disarmed *A. tumefaciens* strain, EHA101 with the super binary plasmid from Ishida et al. (1996). The construct used is described in Table 1:

[1] The anamorph (asexual reproductive stage) of this fungus is *Sporotrichum pruinosum*. While conventions associated with naming of fungi have changed over the years, this document will use this name (*P. chrysosporium*) for this white rot fungus.

Table 1: Genetic Elements and Their Functions in Infinite Enzymes MnP-producing corn.

Genetic Element	Location in Plasmid	Function (Reference)
T-DNA regions		
Right Border Region	10,403-10,427	DNA region from *Agrobacterium tumefaciens* containing the Right Border sequence used for transfer of the T-DNA (Depicker et al., 1982; Zambryski et al., 1982)
Intervening sequence	10,428-10,612	Sequence used in DNA cloning
Globulin-1 promoter	1-1442	DNA region from *Zea mays* globulin-1 gene (1.4 kb) (Belanger and Kriz 1991) AH001354.2
Intervening Sequence	1443	Sequence used in DNA cloning
Barley Alpha Amylase Signal Sequence	1444-1516	Alpha amylase signal sequence from barley, *Hordeum vulgare* (Rogers, 1985). ABBO1247.1
Manganese peroxidase gene	1516-2589	Manganese peroxidase gene from *Phanerochaete chrysosporium*—a white rot fungus. (Tien and Kirk, 1985) J04980.1
Intervening Sequence	2590-2596	Sequence used in DNA cloning
Pin II terminator	2597-2852	Protease inhibitor II gene terminator from potato, *Solanum tuberosum* (An et al., 1989) X04118.1
Intervening Sequence	2853-2961	Sequence used in DNA cloning
35S promoter	2962-3503	Cauliflower mosaic virus promoter for the 35S rna. (Franck et al., 1980) NC_001497.2
Intervening Sequence	3504-3523	Sequence used in DNA cloning
Maize optimized PAT	3524-4075	Phosphinothricin acetyl transferase from *Streptomyces viridochromogenes* (Strauch et al., 1988) WP_003988626.1
Intervening sequence	4076-4093	Sequence used in DNA cloning
35S terminator	4094-4288	Cauliflower mosaic virus terminator for the 35S rna. (Franck et al., 1980) NC_001497.2
Intervening Sequence	4289-4355	Sequence used in DNA cloning
Left Border Region	4356-4380	DNA region from *Agrobacterium tumefaciens* containing the Left Border sequence used for transfer of the T-DNA (Depicker et al., 1982; Zambryski et al., 1982)
Vector Backbone		
Vector	4381-10,402	Sequence used in DNA cloning; spectinomycin resistance; origin of replication;

The plasmid map (Figure 1) used for the transformation is found on the following page.

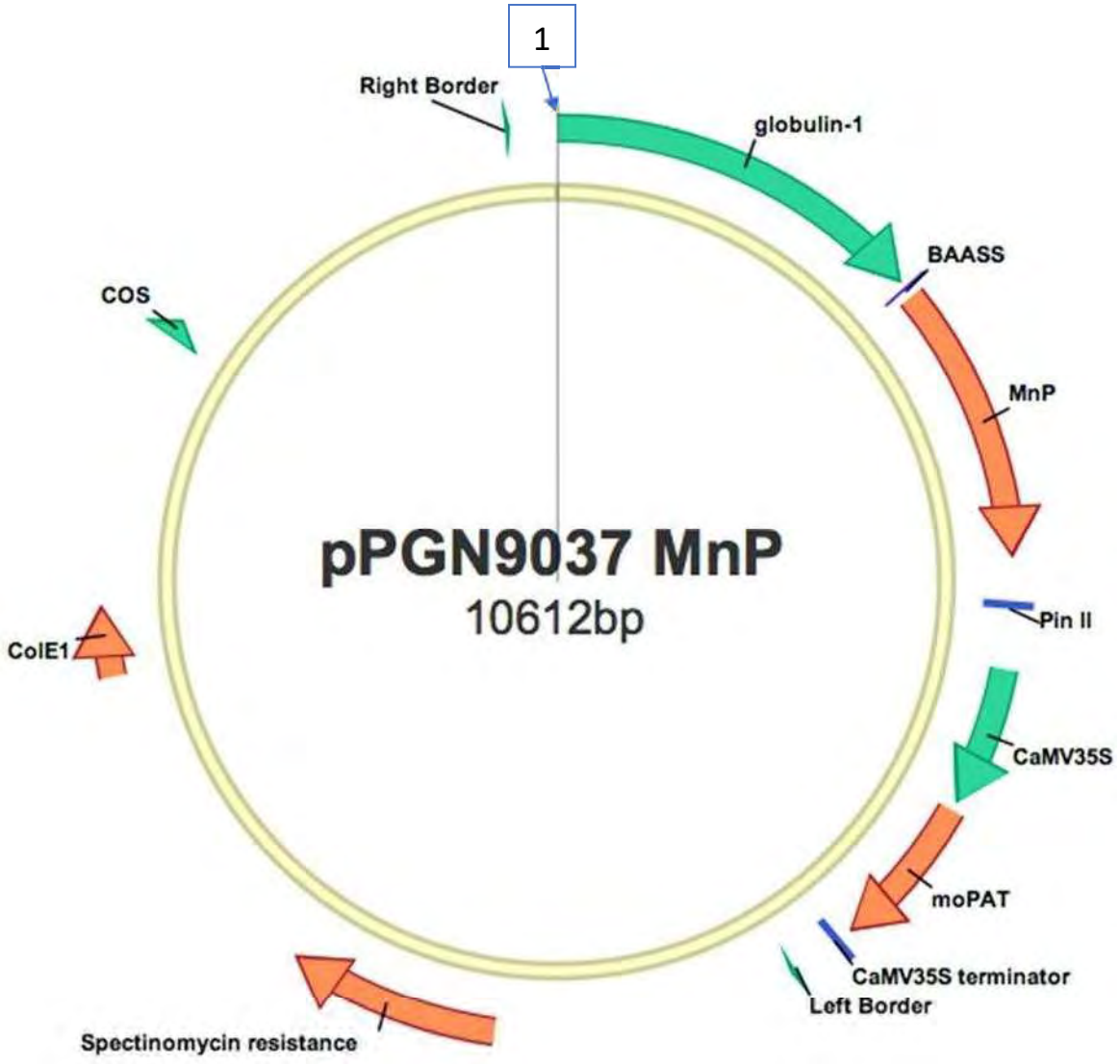

Figure 1 Plasmid map of pPGN9037 MnP

Expression of the MnP protein

As intended, the trait expressed in these plants is identified as embryo-preferred production of manganese peroxidase. This is accomplished by use of the *Zea mays* globulin-1 promoter in combination with the barley alpha amylase signal sequence which further targets production to the cell walls of embryo tissues. This construct has been shown to be highly effective for this purpose (Hood, et. al, 2003). IE has not noted production of MnP in other tissues that have been tested (i.e., leaf, root, stem, endosperm) (Infinite Enzymes, unpublished). Expression of MnP in other tissues or times of plant development, other than embryo growth and development, is not expected. Secondarily, constitutive production of PAT using the cauliflower mosaic virus

35S promoter results in plant tolerance to glufosinate ammonium herbicides.

IE would not expect this event to have impacts on non-target organisms because it is not a plant-incorporated protectant, nor is the trait expressed to a measurable extent in the roots or other vegetative parts of the plant.

Stability of MnP protein in simulated gastric fluid (pepsin/SGF) and simulated intestinal fluid (pancreatin/SIF)

Infinite Enzymes Inc. provided purified manganese peroxidase protein to Dr. Richard Goodman at the Food Allergy Research and Resource Program (FARRP) at the University of Nebraska for stability testing in pepsin and pancreatin. The detailed study, including materials and methods and references, is found in Appendix II. A summary and conclusion from the study is provided here.

"In this study the MnP enzyme was provided by Infinite Enzymes, Inc. as a freeze-dried protein in 50 mM sodium tartrate, with a final enzyme to powder ratio of 60%. The protein was reconstituted in our laboratory and was tested for stability in a test-tube assay with simulated gastric fluid assay (SGF) with pepsin at a fixed pH of 2. Samples were withdrawn at planned time-intervals up to one hour and immediately neutralized in pH and mixed with Laemmle buffer, then heated at >70C for five minutes for evaluation in SDS-PAGE. A large sample following 60 minutes of digestion by pepsin was neutralized with NaOH and acted as the starting material for a digest assay with intestinal fluid assay (SIF) with pancreatin as the digestion enzyme mixture at pH 7.5. The SIF digestion samples were stopped by addition of the sample to microcentrifuge tube with Laemmle buffer and heated to >70C for 5 mins. The timed interval samples were separated in SDS-PAGE gels, then stained with Coomassie blue to visualize residual bands. Images of both assays were captured from white-light illumination using a UVP Image Analyzer. Additional samples were analyzed by western blot using sera from a rabbit sensitized with MnP.

Digestion of the MnP in SGF was rapid. One clear band was detected at approximately 32 kDa at 0.5 minutes of digestion and relatively faint bands were also detected at 15 kDa and less than 10 kDa at that time. By 2 minutes the 32 and 15 kDa bands were not visible. A faint and diffuse band was visible at approximately 10 kDa until 30 minutes when it was reduced to being undetectable. The conclusion is the protein was digested by more than 90% by 2 minutes. Results of digestion in SIF are not interpretable or relevant as there was no detectable MnP residual protein from the 60 min SGF digestion which produced the starting materials for the SIF assay. The bands visible after staining the SIF gel are multiple bands of pancreatin proteins visible without MnP. The western blot was performed at the end of the study to show detection of the MnP protein.

The overall conclusion is that the MnP is rapidly digested in pepsin. This fits the safety profile of a number of proteins that have been tested from Genetically Modified crops, such as Cryl A and CP4 EPSPS in insect resistant and herbicide tolerant maize varieties. This suggests no added risk of food allergy from this protein if maize containing the enzyme was ingested by humans."

Literature and bioinformatics assessment of potential allergy or toxicity risks of MnP protein

Infinite Enzymes Inc. provided manganese peroxidase sequence information to Dr. Richard Goodman at the Food Allergy Research and Resource Program (FARRP) at the University of Nebraska for a literature and bioinformatics assessment of potential allergy or toxicity risks associated with MnP. The detailed study, including materials and methods and references, is found in Appendix III. A summary and conclusion from the study is provided here.

"A literature search was conducted to find any evidence that the protein, or the donor organism, with the enzyme name and with "allergy". The searches were performed with PMC and with PubMed. PubMed was more selective. Finally, only one paper was found with the organism and allergy and that was a case report of a woman with lung infection of an Aspergillosis-like infiltrate, but without reference to the enzyme. Therefore, we found no published scientific reports of allergy to this enzyme from this species. However, using the older species name, *Sporotrichum pruinosum*, a number of publications were found with the inclusion of "toxin" and "toxic" and a few for allergy and allergen. Few papers describe human disease associated with this species or the protein and in no case was it clearly associated with the protein. The protein was noted for detoxification of mycotoxins.

A bioinformatics sequence search was performed with the amino acid sequence (AA) of the MnP protein which was provided by the sponsor as it is expressed in the transformed maize line. It was compared to the sequences of allergens in the www.AllergenOnline.org database using both full-FASTA and sliding 80 AA window searches. The mature protein did not match any protein identified as an allergen by sliding 80mer either in a batch protein comparison on the Holland Computer system super-computer loaded with the AllergenOnline.org version 21 database, or on the public website www.AllergenOnline.org.

The sequence was also compared to the AA sequences of proteins from sequences identified in the NCBI Protein database using keyword searches to identify accession numbers of proteins suspected of having toxic or toxin like activity. The accession number lists for toxins (10,329 sequences) and toxic (9782 sequences) identified in the NCBI Protein database were loaded into the Holland Computing Center server at the University of Nebraska on 23 August 2021 and compared to those sequences by BLASTP version 2.10.0 to identify matches based on high sequence identity and small E scores to find possibly important matches. No sequences were identified that matched the MnP to toxins or allergens using either BLASTP or FASTA 35 or 36 at 35% identity. Thus, there is little risk associated with this protein for human consumers."

Overall conclusions

In using the criteria recommended by the Codex Alimentarius Commission for food safety (2003 and 2009), to test the stability of proteins to digestion, Dr. Goodman found that there are no added risks from the possible consumption of MnP protein from transgenic maize. The addition of testing for stability in SIF did not add to, or detract from the conclusions of the stability in pepsin test. Additionally, from his literature and bioinformatics research, Dr. Goodman found that there are no

added risks from the consumption of this protein considering our current understanding of allergens or possible cross-reactivity for allergy or any role in toxicity or anti-nutrient activity.

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Appendix I

Peroxidases in corn

Peroxidases of various sorts are present in most living organisms. They are particularly abundant in plants and fungi. They perform oxidation-reduction reactions important for moving electrons to form or break molecular bonds.

Twenty-three categories of peroxidases are described in ExplorEnz—The Enzyme Database (https://www.enzyme-database.org/). They vary somewhat in their pH optima, temperature optima, and native substrates. However, they all reduce peroxides, particularly H_2O_2. The vast majority has a heme group in the active site. A recent report even demonstrated the safety of a fungal MnP in the clarification of juice for the food industry (Manavalan et al., 2015).

In 1975, maize was shown to have a minimum of 13 categories of peroxidases (Brewbaker and Hasegawa, 1975). All of them are characterized by having a heme group in the active site. All tissues surveyed contained one or more of the types of peroxidases, including all seed tissues. In 2003, new peroxidases that are membrane bound were identified in maize root tissue (Mika and Lüthje, 2003). The most recent work on peroxidases in maize in the past 10 years has been to characterize individual genes and alleles.

The diversity and abundance of peroxidases in maize tissues and the safety of MnP for food applications justify the consideration of a Mn peroxidase grow out in a traditional permit condition.

EC	**1.11.1.1**
Accepted name:	NADH peroxidase
Reaction:	NADH + H^+ + H_2O_2 = NAD^+ + **2** H_2O
EC	**1.11.1.2**
Accepted name:	NADPH peroxidase
Reaction:	NADPH + H+ + H2O2 = NADP+ + 2 H2O
EC	**1.11.1.3**
Accepted name:	fatty-acid peroxidase
Reaction:	palmitate + **2** H_2O_2 = pentadecanal + CO_2 + **3** H_2O
EC	**1.11.1.4**
Transferred entry :	now EC 1.13.11.11 tryptophan 2,3-dioxygenase
EC	**1.11.1.5**

Accepted name:	cytochrome-*c* peroxidase
Reaction:	**2** ferrocytochrome *c* + H_2O_2 = **2** ferricytochrome *c* + **2** H_2O
EC	**1.11.1.6**
Accepted name:	catalase
Reaction:	**2** H_2O_2 = O_2 + **2** H_2O
EC	**1.11.1.7**
Accepted name:	peroxidase
Reaction:	**2** phenolic donor + H_2O_2 = **2** phenoxyl radical of the donor + **2** H_2O
EC	**1.11.1.8**
Accepted name:	iodide peroxidase
Reaction:	(1) **2** iodide + H_2O_2 + **2** H^+ = diiodine + **2** H_2O
	(2) [thyroglobulin]-L-tyrosine + iodide + H_2O_2 = [thyroglobulin]-3-iodo-L-tyrosine + **2** H_2O
	(3) [thyroglobulin]-3-iodo-L-tyrosine + iodide + H_2O_2 = [thyroglobulin]-3,5-diiodo-L-tyrosine + **2** H_2O
	(4) **2** [thyroglobulin]-3,5-diiodo-L-tyrosine + H_2O_2 = [thyroglobulin]-L-thyroxine + [thyroglobulin]-aminoacrylate + **2** H_2O
	(5) [thyroglobulin]-3-iodo-L-tyrosine + [thyroglobulin]-3,5-diiodo-L-tyrosine + H_2O_2 = [thyroglobulin]-3,5,3′-triiodo-L-thyronine + [thyroglobulin]-aminoacrylate + **2** H_2O
EC	**1.11.1.9**
Accepted name:	glutathione peroxidase
Reaction:	**2** glutathione + H_2O_2 = glutathione disulfide + **2** H_2O
EC	**1.11.1.10**
Accepted name:	chloride peroxidase
Reaction:	RH + chloride + H_2O_2 = RCl + **2** H_2O
EC	**1.11.1.11**
Accepted name:	L-ascorbate peroxidase
Reaction:	**2** L-ascorbate + H_2O_2 + **2** H^+ = L-ascorbate + L-dehydroascorbate + **2** H_2O (overall reaction)

	(1a) **2** L-ascorbate + H_2O_2 + **2** H^+ = **2** monodehydroascorbate + **2** H_2O
	(1b) **2** monodehydroascorbate = L-ascorbate + L-dehydroascorbate (spontaneous)
EC	**1.11.1.12**
Accepted name:	phospholipid-hydroperoxide glutathione peroxidase
Reaction:	**2** glutathione + a hydroperoxy-fatty-acyl-[lipid] = glutathione disulfide + a hydroxy-fatty-acyl-[lipid] + H_2O
EC	**1.11.1.13**
Accepted name:	manganese peroxidase
Reaction:	**2** Mn(II) + **2** H^+ + H_2O_2 = **2** Mn(III) + **2** H_2O
EC	**1.11.1.14**
Accepted name:	lignin peroxidase
Reaction:	(1) 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol + H_2O_2 = 3,4-dimethoxybenzaldehyde + 2-methoxyphenol + glycolaldehyde + H_2O
	(2) **2** (3,4-dimethoxyphenyl)methanol + H_2O_2 = **2** (3,4-dimethoxyphenyl)methanol radical + **2** H_2O
EC	**1.11.1.15**
Accepted name:	peroxiredoxin
Reaction:	**2** R′-SH + ROOH = R′-S-S-R′ + H_2O + ROH
EC	**1.11.1.16**
Accepted name:	versatile peroxidase
Reaction:	(1) 1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol + H_2O_2 = 4-hydroxy-3-methoxybenzaldehyde + 2-methoxyphenol + glycolaldehyde + H_2O
	(2) **2** manganese(II) + **2** H^+ + H_2O_2 = **2** manganese(III) + **2** H_2O
EC	**1.11.1.17**
Accepted name:	glutathione amide-dependent peroxidase
Reaction:	**2** glutathione amide + H_2O_2 = glutathione amide disulfide + **2** H_2O
EC	**1.11.1.18**

Accepted name:	bromide peroxidase
Reaction:	RH + HBr + H_2O_2 = RBr + **2** H_2O
EC	**1.11.1.19**
Accepted name:	dye decolorizing peroxidase
Reaction:	Reactive Blue 5 + **2** H_2O_2 = phthalate + 2,2′-disulfonyl azobenzene + 3-[(4-amino-6-chloro-1,3,5-triazin-2-yl)amino]benzenesulfonate + **2** H_2O
EC	**1.11.1.20**
Accepted name:	prostamide/prostaglandin $F_{2\alpha}$ synthase
Reaction:	thioredoxin + (5Z,9α,11α,13*E*,15*S*)-9,11-epidioxy-15-hydroxy-prosta-5,13-dienoate = thioredoxin disulfide + (5Z,9α,11α,13*E*,15*S*)-9,11,15-trihydroxyprosta-5,13-dienoate
EC	**1.11.1.21**
Accepted name:	catalase-peroxidase
Reaction:	(1) donor + H_2O_2 = oxidized donor + **2** H_2O
	(2) **2** H_2O_2 = O_2 + **2** H_2O
EC	**1.11.1.22**
Accepted name:	hydroperoxy fatty acid reductase
Reaction:	a hydroperoxy fatty acid + NADPH + H^+ = a hydroxy fatty acid + $NADP^+$ + H_2O
EC	**1.11.1.23**
Accepted name:	(*S*)-2-hydroxypropylphosphonic acid epoxidase
Reaction:	(*S*)-2-hydroxypropylphosphonate + H_2O_2 = (1*R*,2*S*)-1,2-epoxypropylphosphonate + **2** H_2O

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Appendix II

Study Title

Testing the stability of the manganese-dependent peroxidase from *Phanerochaete chrysosporium* in SGF and SIF

Author

Richard E. Goodman

Study Completed On

9 February 2022

Performed by

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Laboratory Project ID

Study Number: REG-IE in SGF&SIF-2021

Summary

Infinite Enzymes, Inc., has developed a transgenic maize that expresses the manganese-dependent peroxidase (MnP) enzyme from the Basidiomycete fungus, *Phanerochaete chrysosporium*. The product is intended to provide production of large quantities of the enzyme that will be used for environmental detoxification as a remedial treatment. There is no current plan to include grain from this transgenic plant in food or feed. The evaluation of potential risks of allergy are intended to verify that if the maize did enter the food chain, it would not put allergic consumers at risk. The maize line is currently being produced under USDA-APHIS permit.

In this study the MnP enzyme was provided by Infinite Enzymes, Inc. as a freeze-dried protein in 50 mM sodium tartrate, with a final enzyme to powder ratio of 60%. The protein was reconstituted in our laboratory and was tested for stability in a test-tube assay with simulated gastric fluid assay (SGF) with pepsin at a fixed pH of 2. Samples were withdrawn at planned time-intervals up to one hour and immediately neutralized in pH and mixed with Laemmle buffer, then heated at >70C for five minutes for evaluation in SDS-PAGE. A large sample following 60 minutes of digestion by pepsin was neutralized with NaOH and acted as the starting material for a digest assay with intestinal fluid assay (SIF) with pancreatin as the digestion enzyme mixture at pH 7.5. The SIF digestion samples were stopped by addition of the sample to microcentrifuge tube with Laemmle buffer and heated to >70C for 5 mins. The timed interval samples were separated in SDS-PAGE gels, then stained with Coomassie blue to visualize residual bands. Images of both assays were captured from white-light illumination using a UVP Image Analyzer. Additional samples were analyzed by western blot using sera from a rabbit sensitized with MnP.

Digestion of the MnP in SGF was rapid. One clear band was detected at approximately 32 kDa at 0.5 minutes of digestion and relatively faint bands were also detected at 15 kDa and less than 10 kDa at that time. By 2 minutes the 32 and 15 kDa bands were not visible. A faint and diffuse band was visible at approximately 10 kDa until 30 minutes when it was reduced to being undetectable. The conclusion is the protein was digested by more than 90% by 2 minutes.

Results of digestion in SIF are not interpretable or relevant as there was no detectable MnP residual protein from the 60 min SGF digestion which produced the starting materials for the SIF assay. The bands visible after staining the SIF gel are multiple bands of pancreatin proteins visible without MnP. The western blot was performed at the end of the study to show detection of the MnP protein.

The overall conclusion is that the MnP is rapidly digested in pepsin. This fits the safety profile of a number of proteins that have been tested from Genetically Modified crops, such as Cry1 A and CP4 EPSPS in insect resistant and herbicide tolerant maize varieties. This suggests no added risk of food allergy from this protein if maize containing the enzyme was ingested by humans.

Study Number: REG-IE in SGF&SIF-2021

Testing the stability of the manganese-dependent peroxidase from *Phanerodontia chrysosporium* in SGF and SIF

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Study Start Date: 1 November 2021

Study Completion Date: 9 February 2022

Records Retention: All study specific raw data and a copy of the final report will be retained by Richard E. Goodman

Signature of Final Report Approval:

9 February 2022

Principle Investigator: Richard E. Goodman — Date

Table of Contents

Abbreviations and Definitions

aa	Amino acid
$A_{280\ nm}$	Absorbance of light at a wavelength of 280 nm
D0-60	Digestion samples (protein extract plus pepsin) from time 0 (quenched prior to digestion) and at time 60 min.
EO	Experimental control pepsin without MnP, time 0
E60	Experimental control pepsin without MnP, 60 min.
E-SIF	Pancreatin in SIF
Hb	Acidified 2% hemoglobin
HRP	Horseradish peroxidase
kDa	kilodalton
LOD	Lower limit of detection
LSB	Laemmli solution buffer
mA	milliampere
mg	milligram
mL	milliliter
mM	millimolar
na	Not applicable
Mg	microgram
gL	microliter
Na	Not applicable
ng	nanogram
NFDM	Nonfat dried milk
PO	Experimental control MnP without pepsin, time 0
P60	Experimental control MnP without pepsin, 60 min.
P120	Experimental control protein without enzyme 120 min
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
Pmix	Protein without pancreatin
PVDF	Poly vinylidene fluoride membrane
P/N	Product number, same as catalog number
Pl/10	Experimental Control protein rice leaf protein 10% sample for gels
R^2	Square of the correlation coefficient
SDS	Sodium dodecyl sulfate
SGF	Simulated gastric fluid (without pepsin)
SOP	Standard operating procedure
T	Time point
TCA	Trichloroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
vdc	volts, direct current
v/v	solute volume to solution volume
w/v	solute weight to solution volume

1.0 Introduction

Infinite Enzymes, Inc. developed a maize variety *(Zea mays)* that produces the manganese (Mn) Peroxidase enzyme originally produced in a white rot fungus, *Phanerochaete chrysosporium.* The genetic construct was produced and inserted into a maize line using a recombinant *Agrobacterium tumefaciens* strain EHA101. The genetic construct was described and submitted to the USDA APHIS Biotechnology Regulatory Services in September 2021. As described, maize naturally expresses a number of peroxidases, however, this added gene allows high level production of an enzyme that has been used for environmental clean-up of toxic waste contamination. The product is intended for environmental remediation, and it is not intended to be a food or feed product.

2.0 Purpose. The purpose of this study is to perform an evaluation of the potential stability of the Manganese Peroxidase (MnP) enzyme to consider potential risks of allergenicity of the enzyme produced in a transgenic maize line by Infinite Enzymes, Inc. of Jonesboro, AR. The rationale for the evaluation is to determine whether this maize did get incorporated in food or feed, would it pose a risk of food allergy or would it behave like an endogenous maize protein?

3.0 Methods. The evaluation for potential food risks of allergy for a transgenic food crop include evaluation of potential digestion of the protein in a simulated gastric fluid (SGF) model using porcine pepsin as the proteolytic enzyme. An additional digestion assay is performed for some proteins using simulated intestinal fluid (SIF) with porcine pancreatin as the mixed digestion enzyme. The primary digestion assay has always focused on the action by pepsin (Astwood et al., 1996; Bannon et al., 2002; Thomas et al., 2004), with refinement of the assay by Ofori-Anti et al. (2008). Stability of a number of proteins in SIF assays have been reported by Fu et al. (2002) and Toomer et al., (2013). The methods for stability in SGF follow Thomas al, 2004 and Ofori-Anti et al., 2008). The method for SIF follows that described by Toomer et al., (2013) with extended digestion sampling up to 2 hours. An additional study was performed by searches in scientific literature for allergic reactivity and by bioinformatics comparisons of the amino acid sequence of any newly expressed proteins to the sequences of known allergens that will be presented in another report.

4.0 Materials and Results

The MnP protein was prepared and submitted to us by Infinite Enzymes as a lyophilized powder. The sample was dissolved in distilled water and the concentration of the protein was determined by measuring by a 2-D Quant assay kit (80-6483-56, lot #0618-02). The estimated stock concentration was 14.04 mg/ml using the standard BSA as a reference. The solution was frozen at -20C until used in specific tests.

A sample of raw soybean flour (0.5 mg) was extracted in 5 ml of 1 x phosphate buffered saline (PBS) at pH 7.4 at room temperature for two hours. The sample was clarified by centrifugation at 3,000 x g and 4°C for 20 mins. The clarified solution was filtered through 0.45 micron sterile filter and aliquoted and frozen at -20°C until used. The concentration

was estimated to be 2.5 micrograms per microliter. This sample was used as a negative control for western blots along with an extract of kidney bean and peanut.

Sera from a rabbit sensitized with MnP and after boosting, blood was collected and serum separated from the red blood cells. The serum was provided to us by Infinite Enzymes on 4 January 2022.

4.1 Dilution series demonstrating the limit of detection of MnP in SDS-PAGE reducing gel with proteins stained by Coomassie Blue R-250 after separation. The concentration of MnP stock was 14.04 mg/ml. It was diluted as needed to show detection limit and for preparation of digestions. Figure 1 shows the stained gel with MnP appearing as two primary bands at 34 and 39 kDa. Lane 4 represents 100%. Lane 2 represents 200%, lane 9 represents 10% of the target digestion sample. The digestions were set up with a target protein content of 1.47 micrograms per well in the final SDS-PAGE gel.

4.2 Pepsin activity measurement. The ability of pepsin A to digest proteins in conditions used for this assay was tested by digestion of bovine blood hemoglobin Sigma-Aldrich H2625. Worthington states the activity of pepsin on each lot of LS003319. We test the activity within 24 hours of performing the assay, by digesting fixed amounts of hemoglobin at the pH of the assay. The activity per mg of protein is calculated and diluted in SGF reaction buffer with 35 mM NaCl and HC1 with 0.084 N), adjusted to pH 1.2 or pH 2 as needed. The reactions are carried out in test tubes in a 37°C water bath with blank and test samples in triplicate. After 5 mins of digestion, the residual protein is precipitated with trichloroacetic acid. Free amino acids are measured in a spectrophotometer and the amount is calculated to verify the activity of pepsin is within 20% of the stated activity. The activity was determined for these assays and was within the limits in our protocol.

4.3 Pepsin digestion (SGF). Each sample gel has 15 wells. The samples include protein with and without pepsin incubated for 0 mins and 60 mins at 37°C, pepsin alone for 0 and 60 mins at 37°C. One sample is protein plus pepsin that was held with heat killed pepsin (no digestion) and timed samples were removed at 0.5 min, 2 min, 5 min, 10 min, 20 min, 30 min and 60 min. At each time point the sample is removed and added to pH 7 buffer with Laemmli buffer and heat treated to stop digestion. A sample of 200 microliters of undigested MnP was removed from the 60-minute sample and neutralized for digestion in simulated intestinal fluid (SIF).

Another control sample is a 10% protein sample with quenched pepsin that was included before the last marker protein well. Molecular weight BioRad precision plus markers are included in the outer two lanes in the gel. At the end of digestion ten microliter samples in Laemmli buffer were heated to 90°C for five minutes before a rapid cool to room temperature and loading in a 10-20% SDS-PAGE gel for separation by electrophoresis. Electrophoresis was accomplished with 125 vDC for approximately 1.5 hours and stopped when the loading buffer blue mark was approximately 1 cm above the bottom of the gel. The plastic gel holder was pried open, and the gel was placed in Coomassie R-250 stain which contains fixative for two hours. The gel was then destained in BioRad

destaining solution over-night with two changes and with kimwipes to absorb excess stain. A photographic image was taken using white light on the UVP Image station, The image was captured and appears as Figure 2. The residual stopped Laemmli samples that were not separated by electrophoresis were stored at -20°C and were separated in another gel for western blotting with rabbit serum against MnP.

4.4 Simulated Intestinal Fluid (SIF) digestion. The protein sample from SGF from 60 minutes of digestion by pepsin was quenched at pH 7.5 and used for SIF digestion. Samples that were included were time zero MnP and time 120 minutes of SIF of MnP. In addition, pancreatin mix with the post-SGF digested sample from time zero, from 2 mins, five mins, 10 mins, 20 mins, 30 mins, 60 mins and 120 mins were included. A 10% residual MnP from 60 mins in SGF was used as a control. Pancreatin at 0 mins and pancreatin at 120 mins were controls. Marker lanes were on both outside lanes. The diminished bands in lane 14 show that overall digestion of pancreatin proteins over two hours in Figure 3. Since there were no residual bands of MnP at the start of the SIF assay, it is not possible to judge the stability of the protein with pancreatin alone.

4.5 Western Blots to verify MnP. Since the MnP stained gel had multiple bands, Infinite Enzymes provided serum from a rabbit they had injected with purified MnP to produce specific IgG. Samples of the MnP and digestion products stored at -20°C were separated on a new SDS-PAGE Invitrogen 10-20% gel using reducing Laemmli buffer, following heating at 90°C before sample application to a gel for stained gel detection and to a gel for transfer to PVDF for western blot. Samples included BioRad Precision Plus protein stain in lanes 1 and 14. Lane 2 included a sample of 2 micrograms of maize grain extract. Lane 3 was from concentrated MnP from Infinity Enzyme (6 micrograms). Lane 4 was empty. Lane 5 was from 200% MnP. Lane 6 was diluted 10% sample. Lane 7 was from 100% MnP protein. Lane 8 was empty. Lane 9 was from kidney bean extract at 2 micrograms of extract. Lane 10 was empty. Lane 11 was from 1 micrograms of soybean extract. Lane 12 was empty. Lane 13 was from 2 micrograms of peanut extract and lane 14 was BioRad Precision plus protein markers. Figure 4 is a stained gel of the separated proteins. Figure 5 is the western blot performed with rabbit serum followed by four washes with TBST to remove unbound serum IgG and then incubation of horse radish peroxidase (HRP) labeled goat anti-rabbit IgE and then 1:1 mixed A and B substrates. The damp PVDF membrane was inserted in the UVP detector and adjusted for image capture. The ECL image was captured and selected for presentation in Figure 5.

5.0 Conclusions

In using the criteria recommended by the Codex Alimentarius Commission for food safety (2003 and 2009), to test the stability of proteins to digestion, I find that there are no added risks from the possible consumption of MnP protein from transgenic maize. The addition of testing for stability in SIF did not add to, or detract from the conclusions of the stability in pepsin test.

6.0 References

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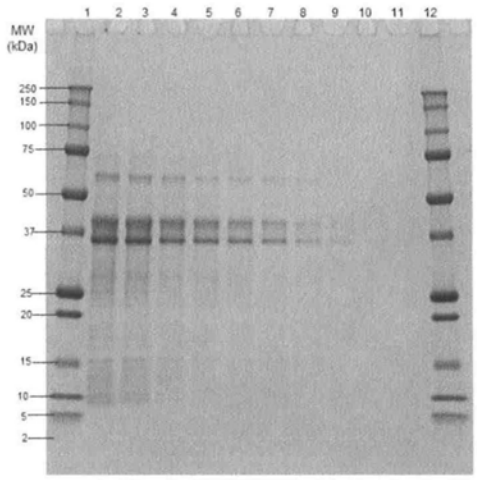
9.0 Figures

Figure 1. Limit of detection of MnP in stained gel. Samples of the purified MnP were diluted and separated in a reduced denaturing gel with Laeemli buffer and beta-mercaptoethanol. The gel was stained with Coomassie blue R250 with acid fixing agent. Bands were detectable to less than 0.07 micrograms of protein.

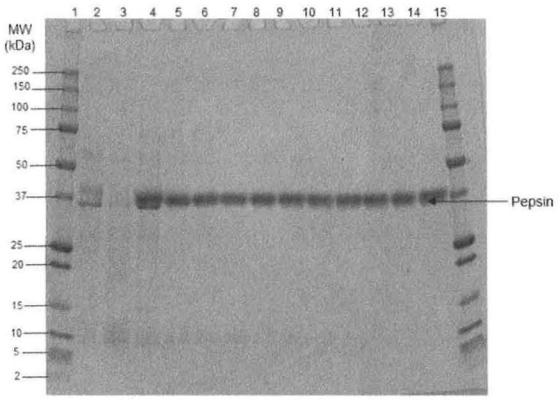


Coomassie R-250 stained SDS-PAGE gel showing Limit of Detection of manganese dependent peroxidase. Proteins were separated by SDS-PAGE using a 10->20%Tris-Glycine Invitrogen gel in a constant voltage of 200 volts for 40 minuets.

Limit of Detection of manganese dependent peroxidase

Lane	Description	Quantity of protein Loaded/well
1	Molecular weight marker	
2	200% manganese dependent peroxidase extract	2.94 pg
3	150% manganese dependent peroxidase extract	2.21 pg
4	100% manganese dependent peroxidase extract	1.47 pg
5	80% manganese dependent peroxidase extract	1.18 pg
6	60% manganese dependent peroxidase extract	0.88 pg
7	40% manganese dependent peroxidase extract	0.59 pg
8	20% manganese dependent peroxidase extract	0.29 pg
9	10% manganese dependent peroxidase extract	0.15 pg
10	5% manganese dependent peroxidase extract	0.07 pg
11	2.5% manganese dependent peroxidase extract	0.04 pg
12	Molecular weight marker	

Figure 2. Stability of MnP in the SGF assay. The MnP protein is shown mostly as a double band at 37 and another more than 37 kDa. The control at lane 3 shows the MnP is mostly digested at 37°C after 60 minutes even without full digestion. Lane 5 shows marked reduction in MnP and lane 6 at 2 mins suggests nearly complete digestion of the protein. The 10% residual sample in lane 14 is barely detectable. This suggests that the protein is not stable in pepsin at 37°C.

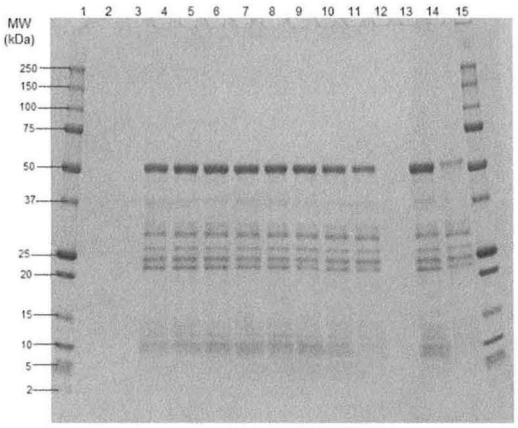


Pepsin digestion of manganese dependent peroxidase proteins in Coomassie Blue R-250 stained gel. Proteins were digested as described in the protocol. The samples were run on a 10-20% Tris Glycine gel under reducing conditions in a constant voltage of 200 volts for 40 minuets. Load per well was 10 µl (1.47µg of protein).

Pepsin digestion of manganese dependent peroxidase at pH 2

Lane	Description	Incubation time (min)
1	Molecular weight marker in kDa	na
2	Peroxidase without pepsin (control)	0
3	Peroxidase without pepsin (control)	60
4	Peroxidase sample	0
5	Peroxidase sample	0.5
6	Peroxidase sample	2
7	Peroxidase sample	5
8	Peroxidase sample	10
9	Peroxidase sample	20
10	Peroxidase sample	30
11	Peroxidase sample	60
12	Pepsin only	0
13	Pepsin only	60
14	Peroxidase sample, 10% protein with quenched pepsin	0
15	Molecular weight marker in kDa	na

Figure 3. Stability of the residual protein from SGF in the SIF assay. Lanes 2 and 3 show that there are no residual proteins following digestion by SGF or SIF.



Pancreatin digestion of manganese dependent peroxidase proteins in Coomassie Blue R-250 stained gel. Proteins were digested in pancreatin, following 60 min digestion in pepsin and neutralizing pH at 7.5. The samples were run on a 10-20% Tris Glycine gel under reducing conditions in a constant voltage of 200 volts for 40 minuets. Load per well was 10 µl (1.47µg of protein).

Pancreatin digestion of manganese dependent peroxidase at pH 7.5

Lane	Description	Incubation time (min)
1	Molecular weight marker in kDa	na
2	Peroxidase after pepsin digestion, without pancreatin (control)	0
3	Peroxidase after pepsin digestion, without pancreatin (control)	120
4	Peroxidase digestion	0
5	Peroxidase digestion	2
6	Peroxidase digestion	5
7	Peroxidase digestion	10
8	Peroxidase digestion	20
9	Peroxidase digestion	30
10	Peroxidase digestion	60
11	Peroxidase digestion	120
12	10% peroxidase after pepsin digestion	0
13	Pancreatin only	0
14	Pancreatin only	120
15	Molecular weight marker in kDa	na

Figure 4. Coomassie stained gel of samples used in Western Blot. Samples in this gel represent proteins extracted from non-transgenic maize, purified MnP protein at four concentrations, proteins from kidney beans, proteins from soybean and proteins from peanut extract. The bands at just above and just below 37 kDa represent the MnP proteins.

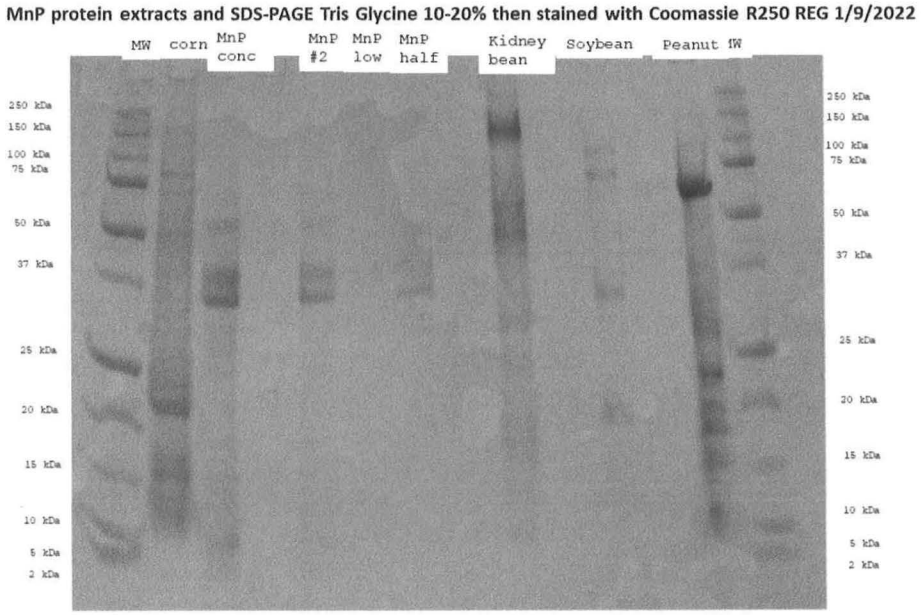
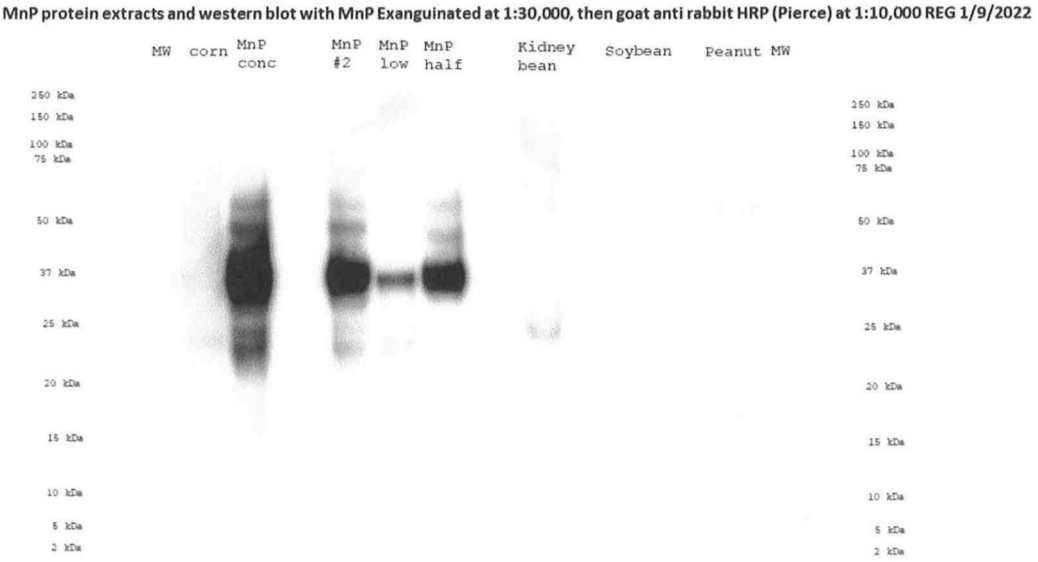


Figure 5. Western blot with serum of rabbit immunized with MnP. Secondary antibody was goat IgG specific for rabbit IgG. The goat antibody was conjugated with horse radish peroxidase. Following exposure and washing, the HRP was detected using substrates that emit light due to HRP activity. The specific binding is to the 37 kDa MnP protein.



Appendix III

Study Title

Literature and bioinformatics analysis of potential allergy or toxicity risks of manganese-dependent peroxidase from *Phanerochaete chrysosporium* expressed in maize

Author

Richard E. Goodman

Study Completed On

9 February 2022

Performed by

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Laboratory Project ID

Study Number: REG-Infinite Enzymes MnP 2021-B

Summary

Infinite Enzymes, Inc., has developed a transgenic maize that expresses the manganese-dependent peroxidase (MnP) enzyme from the Basidiomycete fungus, *Phanerochaete chrysosporium.* The previous scientific name for this species was *Sporotrichum pruinosum* as the imperfect stage of the fungus. The current product is intended to provide production of large quantities of the enzyme that can be used for environmental detoxification as a remedial treatment for mycotoxins and other hazards. This transformed maize is not intended for direct production in food or feed. The evaluation of potential risks of allergy are intended to verify that if the maize did enter the food chain, it would not put consumers allergic to a homologous protein at risk. The maize line is currently being produced under USDA-APHIS permit.

A literature search was conducted to find any evidence that the protein, or the donor organism, with the enzyme name and with "allergy". The searches were performed with PMC and with PubMed. PubMed was more selective. Finally, only one paper was found with the organism and allergy and that was a case report of a woman with lung infection of an Aspergillosis-like infiltrate, but without reference to the enzyme. Therefore, we found no published scientific reports of allergy to this enzyme from this species. However, using the older species name, *Sporotrichum pruinosum,* a number of publications were found with the inclusion of "toxin" and "toxic" and a few for allergy and allergen. Few papers describe human disease associated with this species or the protein and in no case was it clearly associated with the protein. The protein was noted for detoxification of mycotoxins.

A bioinformatics sequence search was performed with the amino acid sequence (AA) of the MnP protein which was provided by the sponsor as it is expressed in the transformed maize line. It was compared to the sequences of allergens in the www.AllergenOnline.org database using both full-FASTA and sliding 80 AA window searches. The mature protein did not match any protein identified as an allergen by sliding 80mer either in a batch protein comparison on the Holland Computer system super-computer loaded with the AllergenOnline.org version 21 database, or on the public website www.AllergenOnline.org.

The sequence was also compared to the AA sequences of proteins from sequences identified in the NCBI Protein database using keyword searches to identify accession numbers of proteins suspected of having toxic or toxin like activity. The accession number lists for toxins (10,329 sequences) and toxic (9782 sequences) identified in the NCBI Protein database were loaded into the Holland Computing Center server at the University of Nebraska on 23 August 2021 and compared to those sequences by BLASTP version 2.10.0 to identify matches based on high sequence identity and small E scores to find possibly important matches. No sequences were identified that matched the MnP to toxins or allergens using either BLASTP or FASTA 35 or 36 at 35% identity. Thus, there is little risk associated with this protein for human consumers.

Study Number:	REG-MnP-2021-B
	Literature and bioinformatics analysis of potential allergy or toxicity risks of manganese-dependent peroxidase from *Phanerochaete chrysosporium* expressed in maize
Facility:	RE Goodman Consulting LLC 8110 Dougan Circle Lincoln, NE 68516 USA
Principle Investigator:	Richard E. Goodman Tel: +1 (402) 417-5549
Study Start Date:	1 October 2021
Study Completion Date:	9 February 2022
Records Retention:	All study specific raw data and a copy of the final report will be retained by Richard E. Goodman.

Signature of Final Report Approval:

9 February 2022

Principle Investigator: Richard E. Goodman — Date

Table of Contents

Figures

Abbreviations and Definitions

aa	Amino acid
AOLv21	http://www.AllergenOnline.com/ database version 21
BLASTP	Algorithm used to find local high scoring alignments between a pair of protein sequences (using databases on Entrez)
Entrez NCBI	A public genetic database maintained by the National Center for Biotechnology Information (NCBI) at the National Institutes of Health, Bethesda, MD. Protein entries in the Entrez search and retrieval system are maintained by the NCBI of the National Institutes of Health (U.S.A.)
FARRP	Food Allergy Research and Resource Program, University of Nebraska
FASTA3	Algorithm used to find local high scoring alignments between a pair or protein sequences (using the AllergenOnline database)
GI	A unique identification number assigned by NCBI to each sequence in the database
HHC	Holland Computing Center, University of Nebraska-Lincoln supercomputer
MnP	Manganese dependent peroxidase enzyme from *Phanerochaete chrysosporium*
PubMed	A public information database of scientific journal articles and abstracts maintained by the National Library of Medicine, National Institutes of Health (U.S.A.)
80mer	Sliding window of 80 amino acids of query protein are compared to AOL v 21 by FASTA

1. **Introduction.** *Phanerochaete chrysosporium* has also been known as *Sporotrichum pruinosum* (Burdsall, 1981). Those two names are synonymous. The organisms are in the Phylum Basidiomycota. These are two life forms of the same species, one a diploid or perfect form (P. *chrysosporium)* and the second a haploid or imperfect form (S. *pruinosum).* This species produces strong lignin degrading enzymes that can be used for degrading woody products. However, it also produces a manganese peroxidase enzyme (MnP) enzyme that has been reported to degrade various mycotoxins. That makes it highly useful for enhancing the safety of food including grain that may have been contaminated by certain mycotoxin producing fungal organisms. The MnP enzyme is induced in the fungus by heat shock (Brown et al., 1993). But obtaining sufficient enzyme from wild-fungi does not provide a reliable source for having available MnP for detoxifying food and feed. The gene for MnP has been cloned into maize for efficient production of the enzyme. It can be purified from maize and used for detoxification purposes or it might be useful to express the protein within maize to inactivate mycotoxins.

   The purpose of this study was to search the literature for evidence that the protein, MnP is an allergen or is sufficiently identical in the amino acid sequence to act as a cross-reactive allergen if people who have existing allergies to a homologue are exposed. This study was conducted using the allergen database (www.AllergenOnline.org version 21) by FASTA alignment or to the NCBI Protein database by BLASTP alignment. The CODEX Alimentarius Commission guidelines for genetically modified plants to identify possible risks of allergenicity (Codex, 2003 as modified and re-published in 2009) provides guidance that is conservative for proteins introduced in genetically engineered or genetically modified organisms. The same criteria has also been used to evaluate some novel foods sources. However, the criteria of >35% identity over 80 amino acids is too stringent for allergen predictions due to evolution of proteins across taxonomically separated species (Abdelmoteleb et al., 2021; Aalberse, 2000). Yet the search can provide guidance for whether additional testing is necessary. Previous studies by our laboratory have been able to evaluate potential risks for genetically engineered crops (Siruguri et al., 2015; Jin et al., 2017). Since the AllergenOnline.org database is updated annually, and new proteins may be identified based on scientific data not considered in selection of proteins in AllergenOnline.org, an additional BLASTP search was used comparing proteins identified by keywords: allergen, or allergy, as well as no keyword in the NCBI protein database. A search was made in the NCBI Protein database for accessions labeled with the terms "allergen" or "allergy". The accession numbers using those criteria were collected and sequences inserted in a file in the Holland Computer Center database supercomputer at the University of Nebraska-Lincoln and the search performed to identify potential allergens.

   In addition, a search was performed for possible high identity matches to toxic proteins from any source based on keyword selection of sequence annotations from the NCBI Protein database. There are no readily accepted criteria for matches to toxins and no substantial toxin database. Therefore, matches are compared to other common proteins in the human body or from common food sources that do not have records of toxicity. The

compiled results are meant to identify potential risks of dietary proteins based on similarities to allergens and toxins.

2.0 Purpose. The purpose of this study is to perform an evaluation of the potential allergenicity and toxicity risks of the proteins identified from the leaves of a proprietary MnP protein produced by maize following transformation with the coding sequence of the MnP protein.

3.0 Methods. The evaluation for potential food risks typically includes a careful search of scientific literature for relevant peer-reviewed studies to evaluate the safety of the gene donor as well as the safety of the proteins encoded by any transferred genes. In addition, computer searches (bioinformatics) are used to evaluate the potential similarity of the encoded protein to any known allergen or toxin by comparisons to those in appropriate databases.

3.1 Scientific literature search strategies. The PubMed database (http://www.ncbi.nlm.nih.gov/pubmed) maintained by the U.S. National Library of Medicine was used as the primary data source for scientific literature on allergy and toxicity. The primary question is whether the source of the gene is a common cause of allergy or toxicity. The data (authors, publication, date and abstracts) from searches were saved to files for review. All publication abstracts were manually reviewed and any likely relevant publications suggesting adverse health risks were investigated further by reading the journal articles.

3.1.1 Search for allergenicity. Searches were performed using the names of the species and "allergy" or "allergen". In addition, the name of the enzyme was added, *"manganese peroxidase"* which helped in specificity.

3.1.2 Search for toxicity. A search was performed using the names of the species AND "toxin" or "toxic". Addition of the enzyme name was also used.

3.2 Amino acid sequences of query proteins. The amino acid sequence of the MnP protein was verified and shared by the study sponsor (Infinite Enzymes) and is shown in Figure 1) The search criteria are described in section 3.3.

3.3 Sequence database search strategies. The AllergenOnline.org version 21 (http://www.allergenonline.org/) and the NCBI Entrez Protein (http://www.ncbi.nlm.nih.gov/BLAST/) databases were used as the protein amino acid data sources for the sequence comparisons for allergens and toxins, respectively. The AllergenOnline database was updated on 14 February 2021 and is maintained by the Food Allergy Research and Resource Program of the University of Nebraska. Protein entries in the Entrez search and retrieval system is compiled and maintained by the NCBI of the National Institutes of Health (U.S.A.). The NCBI Protein database is potentially updated weekly; however, in June 2019 the ability to limit searches by keywords was removed. Therefore, the University of Nebraska-Lincoln Holland Computing Center (HCC) supercomputer uploaded the complete NCBI Protein database

in August 2021. The keyword accession list of sequences in the NCBI Protein database in August 2021 were downloaded using keywords (allergen, allergy, toxin and toxic) and the numbers were entered into the HCC system. The sequences of interest were subsequently tested by BLASTP in the system only against proteins having the accession numbers in each list. The output sequences and history of allergy were evaluated for unique attributes.

Figure 1. Wild-type MnP protein sequence from *Phanerochaete chrysosporium.* Mature protein has removed signal peptide and immature leader sequence (underlined). The mature protein is 292 amino acids after removing the underlined leader sequence.

MAFGSLLAFVALAAITRAAPTAESAVCPDGTRVTNAACCAFIAQDLQETLFQGDCGEDAHEVIRLTFHDAI
A1SQSLGPQAGGGADGS
MLHFPTIEPNFSANNGIDDSVNNLLPFMQKHDTISAADLVQFAGAVALSNCPGAPRLEFMAGRPNTTIPAVE
GLIPEPQDSVTKILQRFEDAGNFSPFEVVSLLASHTVARADKVDETIDAAPFDSTPFTFDTQVFLEVLLKGTG
FPGSNNNTGEVMSPLPLGSGSDTGEMRLQSDFALARDERTACFWQSFVNEQEFMAASFKAAMAKLAILGH
SRSSLIDCSDVVPVPKPAVNKPATFPATKGPKDLDTLTCKALKFPTLTSDPGATETLIPHCSNGGMSCPGVQ
FDGPA

3.3.1 FASTA3 overall search of AllergenOnline.org. The mature protein amino acid sequence of 292 AA was compared to AllergenOnline.org using the public website: http://www.allergenonline.org/databasefasta.shtml. The search was performed as Full FASTA version 35 with default setting of £-value of 1, using a BLOSUM 50 scoring matrix. The overall alignment provides the most likely estimate of potential allergic cross-reactivity and is unlikely if identity matches of less than 50% occur for most of the length of the aligned proteins.

3.3.2 FASTA3 of AllergenOnline.org by 80 aa segments. This short segment search with AllergenOnline.org version 20 is available on our public www.AllergenOnline.org website and is based on the recommendation of Codex (2003). However, as noted in our recent publication (Abdelmoteleb et al, 2021) many of the matches between 35% identity and 50% identity or even higher identity numbers over 80 amino acids are unlikely to demonstrate cross-reactivity as the identity matches are shared across broad taxonomic groups. The search starts at AA 1-80, then 2-81 to the end of the search sequence. The *E* score is set to 1,000 to eliminate limitations of short searching query sequences. The matches that seemed relevant in the full-FASTA match were re-evaluated on the public website using the 80mer matching algorithm.

3.3.3 BLASTP of NCBI Protein database on the HCC server for allergens and toxins. The AA sequence of the *Phanerochaete chrysosporium* MnA protein was searched for matches using the NCBI Protein database for allergens and toxins using keyword limits. The AllergenOnline.org full dataset for version 21 was loaded as search targets. Accession number lists for all proteins identified in NCBI Protein searches for "toxin" and for "toxic" were also loaded in the HCC server to use as the search database for toxicity.

3.3.4 BLASTP of NCBI Entrez without a keyword limit. The BLASTP is available on the NCBI Entrez website (http://www.ncbi.nlm.nih.gov/BLAST/). The current version is BLASTP +2.11.0 (January 2021). A BLASTP search was used comparing the MnP to all proteins. Evaluation of the *E* value, the length of the alignment and the percent identity of any identified match is necessary to judge the significance of any alignment using BLASTP. Default BLASTP parameters were used, with Word size = 6, Expect value =10, Gapcosts =11,1, Matrix = BLOSUM 62, Threshold =21.

4.0 Results and Discussion. The summary results for the PubMed literature search are reported and bioinformatics searches using the various protein sources and search terms, and the amino acid sequences of MnP is also reported.

4.1 PubMed Searches.

4.1.1 Allergen and allergy. The PubMed scientific literature database was searched for evidence that the enzyme (MnP) protein from the species *Phanerochaete chrysosporium* or *Sporotrichum pruinosum,* was performed by multiple searches with combinations of terms to identify possible risks of allergy. The search of PubMed identified only 2 papers that seemed to describe possibly allergy to the species, however upon review one described apparent lung infection and not allergy (Daher et al., 2005), The other described sinobronchial infection, but no proof of allergy to a specific protein (Ogawa et al., 2011). When searching PMC, the results were less specific. The PMC database search is much less selective identifying 110 publications, including those identified in Pubmed. Using the species name and allergen identified 65 publications. More detailed reading of abstracts was required to understand the reason for their selection in PMC.

4.1.2 Toxicity. The search for toxicity using species names and "toxin" or "toxic" identified eight publications that were evaluated by reading the abstract. In turn the publications showed that the species *Phanerochaete chrysosporium* and the enzyme type is able to detoxify a number of compounds that are mycotoxins (Wang J et al., 2011; Wang X et al. 2019; Zeng et al., 2019 and Zeng et al. 2020). On hundred and forty-eight publications were found with the species name and "toxic" in PubMed. However, a review of the abstracts indicated that most of the abstracts identified mycotoxins or other fungal toxins. In the end there were no publications that showed clear toxicity to humans from proteins from this species of fungus.

4.2 Sequence comparison of was compared to allergens and to toxins by FASTA and BLASTP. The amino acid sequence of the mature length of the MnP protein (292 amino acids) was compared to allergens in the AllergenOnline.org version 21 database by three methods. The AllergenOnline.org database includes proteins that the committee of experts agree are at least putative allergens based on serum IgE binding with samples from appropriately characterized subjects. Those proteins that also have demonstrated biological activity of activating basophils or mast cells to release mediators of histamine or beta-hexosaminidase are considered as proven allergens. The designation of proteins as "allergens" or "putative allergens" can be seen in the Browse function on the database

(www.AllergenOnline.org). The user can select an *E* score limit of 10 or of 1 as the allowed variation for accepting alignments. The user can also select the version of FASTA to used, either 35.04 or version 36 (WR Pearson, 2014). These were compared to all known and putative allergens in AllergenOnline.org version 21 using a full-length FASTA alignment search and a sliding window of 80 comparison when needed on the public website (www.allergenonline.org). An eight amino acid matching window search was also performed. Additionally, a BLASTP search was performed against the NCBI database. Interpretation of the results required a comparison without the use of keywords.

4.2.1 Full length FASTA3 with AllergenOnline.org MnP protein AA sequence. The 292 AA sequence of the mature MnP protein was searched in the Full-length search using version 35.04 FASTA as well as version 36 FASTA against the AllergenOnline database version 21. No sequence alignments were identified. The searches were repeated with an *E* score limit of 10 and a few alignments were found that identified matches to proteins in the allergen database ranging from 23% identity to 28% identity over 65 to 84 amino acids in length. All were to profilins, of grass, olive tree, litchi or kiwi. Profilin is a highly conserved protein across many diverse taxa and there are few reports that this protein is a bio active allergen. As noted by Rob Aalberse and me (Aalberse, 2000; Goodman, 2006; Goodman et al., 2008) identities of less than 50% are often not likely to be cross-reactive, while those greater than 70% identity are often cross-reactive in the allergic human subject. As noted in our publication in 2021, different protein types should have more focused, individualized criteria for likely cross-reactivity based on the evolutionary conservation from diverse species (Abdelmoteleb et al. 2021).

4.2.2 Sliding 80-amino acid window FASTA3 vs. AllergenOnline.org database. The mature AA sequence of the MnP protein was compared to AllergenOnline.org version 21 using the sliding 80mer window with version 35.04. No alignments >35% identity matches were found using this comparison (Figure 2).

Figure 2. Search of AllergenOnline.org sliding 80mer window using version 21 of the database. The mature sequence of MnP was searched against AllergenOnline v21. The output is shown here, with no matches > 35% identity.

80mer Sliding Window Search Results

Database	AllergenOnlnie Database v21 (February-14.2021)
Input Query-	>query MLHFPTIEPNFSANNGIDDSVNNLLPFMQKHDTISAADLVQFAGAVALSNCPGAPRLEFM AGRPNTTIPAVEGLIPEPQDSVTKILQRFEDAGNFSPFEWSLLASHTVARADKVDETID AAPFDSTPFTFDTQVFLEVLLKGTGFPGSNNNTGEVMSPLPLGSGSDTGEMRLQSDFALA RDERTACFWQSFVNEQEFMAASFKAAMAKLAILGHSRSSLIDCSDWPVPKPAVNKPATF PATKGPKDLDTLTCKALKFPTLTSDPGATETLIPHCSNGGMSCPGVQFDGPA
Length	292
Number of 80 mers	213
Number of Sequences with hits	0

No Matches of Greater than 35% Identity[7] Found

AllergenOnline Database v21 (February 14, 2021)

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4.2.3 Eight Amino Acid identity match to AllergenOnline.org v21. A search for identity matches of 8 amino acids did not identify any match of 8 amino acids to any allergen.

4.2.4 BLASTP of MnP to the NCBI Protein database for matches to sequences identified as allergens or toxins on the NCBI database. The full-length amino acid sequences of the mature length of the MnP was tested using the BLASTP search algorithm using default criteria of public BLASTP on the Holland Computing Center computer to the NCBI Protein non-redundant database. The database was downloaded to HCC on 23 August 2021. The BLASTP was run on the HCC supercomputer accession sequence limits for "allergen", "allergy", "toxin" and "toxic". The NCBI Protein data entries for each sequence had to contain one of the four terms in defined fields, The accession numbers were collected and the entries downloaded from NCBI Protein on 23 August 2021. The sequence of the mature MnP protein were compared to those by BLASTP. Sequence identities, percent sequence values, alignment lengths and E scores were captured in an Excel file. The data is summarized in Table 1.

Table 1. BLASTP comparison results for mature MnP compared to the protein amino acid sequences limited by the keyword. The mature sequence of 292 AA was used for this search. The four keyword defined entries are listed.

Keyword "Allergen"			
Subject ID	% Seq Identity	Alignment length	E-score
ANT46161.1 ascorbate peroxidase K [Musa acuminata]	27.4	241 AA	1.02 E-12
ANT46164.1 ascorbate peroxidase L [Musa acuminata]	27.0	241 AA	3.9 E-12
KPM11763.1 Sar s 3 allergen (serine protease-like protein 14) [Sarcoptes scabiei]	43.8	32 AA	3.3
KAF7488538.1 Mite allergen Derp 3 [Sarcoptes scabiei]	43.8	32 AA	7.6

Keyword "Allergy"			
EMJ97338.1 adenylate/guanylate cyclase catalytic domain protein [Leptospira sp. B5-022]	29.7	175 AA	1.7 E-7
EIE00929.1 2Fe-2S iron-sulfur cluster-binding domain / adenylate/guanylate cyclase catalytic domain / peroxidase multi-domain protein [Leptospira licerasiae serovar Varillal str. VAR 010]	29.1	175	3.0 E-7
EPG67554.1 adenylate/guanylate cyclase catalytic domain protein [Leptospira wolffii serovar Khorat str. Khorat-H2]	27.2	180 AA	2.3 E-6
EQA38358.1 adenylate/guanylate cyclase catalytic domain protein [Leptospira inadai serovar Lyme str. 10]	24.8	258 AA	2.1 E-4
EPG76428.1 adenylate/guanylate cyclase catalytic domain protein [Leptospira fainei serovar Hurstbridge str. BUT 61	23.7	207 AA	0.002
EQA43839.1 adenylate/guanylate cyclase catalytic domain protein [Leptospira broomii serovar Hurstbridge str. 5399]	24.4	258 AA	0.004
EKOI4483.1 adenylate/guanylate cyclase catalytic domain protein [Leptospira kirschneri str. H1]	28.7	129 AA	0.008
EMK04418.1 adenylate/guanylate cyclase catalytic domain protein [Leptospira kirschneri]	28.9	135 AA	0.008
EMJ94036.1 adenylate/guanylate cyclase catalytic domain protein [Leptospira kirschneri str. JB]	29.3	123 AA	0.008
EMK25426.1 adenylate/guanylate cyclase catalytic domain protein [Leptospira kirschneri serovar Bulgarica str. Nikolaevo]	28.7	129 AA	0.008
Keyword "Toxin"			
KLT39651.1 heme peroxidase [Cutaneotrichosporon oleaginosum]	32.2	180 AA	6.5 E-8
TWQ02467.1 tail fiber protein, partial [Escherichia coli]	29.8	124 AA	1.2
XP_021346173.1 cysteine-rich, acidic integral membrane protein-like [Mizuhopecten yessoensis]	27.5	80 AA	1.3
CRX94760.1 putative RTX-family protein [Yersinia enterocolitical	25	104 AA	6
WP_050161131.1 MARTX multifunctional-autoprocessing repeats-in-toxin holotoxin RtxA [Yersinia enterocolitica]	25	104 AA	6
WP_042663 873.1 MARTX multifunctional-autoprocessing repeats-in-toxin holotoxin RtxA [Yersinia enterocolitica]	25	104 AA	6
WP_115240265.1 MARTX multifunctional-autoprocessing repeats-in-toxin holotoxin RtxA [Yersinia enterocolitica]	25	104 AA	6
WP_032904652.1 MARTX multifunctional-autoprocessing repeats-in-toxin holotoxin RtxA [Yersinia enterocolitica]	25	104 AA	6
WP_020283406.1 MARTX multifunctional-autoprocessing repeats-in-toxin holotoxin RtxA [Yersinia enterocolitica]	25	104 AA	6

RGS29851.1 DUF4825 domain-containing protein [Eubacterium sp. AF22-8LB]	33.3	51 AA	8.7
Keyword "Toxic"			
sp|Q59X94.2|CCPR2_CANAL RecName: Full=Putative heme-binding peroxidase	27.4	259 AA	2E-12
ABS70719.1 peroxisomal ascorbate peroxidase [Avicennia marina]	25.3	241 AA	3.3 E-12
sp|Q4PD66.1|CCPR2_USTMA RecName: Full=Putative heme-binding peroxidase	25.4	248	1.2 E-l 1
sp|Q6BIB1.3|CCPR2_DEBHA RecName: Full=Putative heme-binding peroxidase	26.7	251 AA	4.7 E-10
sp|Q4WLG9.1|CCPR2_ASPFU RecName: Full=Putative heme-binding peroxidase	26.2	248	5.4 E-10
sp|Q4HWQ2.1|CCPR2_GIBZE RecName: Full=Putative heme-binding peroxidase	24.0	279 AA	5.8 E-10
sp|Q6C0Z6.1|CCPR_YARLI RecName: Full=Cytochrome c peroxidase, mitochondrial; Short=CCP; Flags: Precursor	26.4	242 AA	6.8 E-10
CAK38671.1 unnamed protein product [Aspergillus niger]	22.9	278 AA	8.8 E-10
sp|Q6CAB5.1|CCPR2_YARLI RecName: Full=Putative cytochrome c peroxidase, mitochondrial; Short=CCP; Flags: Precursor	23.5	272 AA	2.4 E-9
sp|Q6C7Ul.l|CCPR3_YARLI RecName: Full=Putative heme-binding peroxidase	25	252	2.6 E-9

4.3 Bioinformatics summary for the MnP protein of Infinite Enzymes. The literature searches for evidence of allergy or toxicity to this protein from *Phanerochaete chrysosporium* (or *Sporotrichum pruinosum)* did not identify any expected risk of allergy or toxicity associated with this species or with the MnP protein. The AA sequence comparisons were performed using the 292 AA sequence of the mature protein provided by Infinite Enzymes as representing their product. The primary searches were against the www.allergenonline.org version 21 database using the public website with FASTA 35.04 and repeated with sliding 80 AA window comparisons. I included the option of an identity match to any segment of 8 amino acids to any of the allergens in our database. There were no matches. We also performed a BLASTP comparison to the NCBI database using sequences collected based on keyword limits from the NCBI Protein database. All matches in that case (Table 1) were low identity and are not likely to represent a cross-reactive risk. The BLASTP search also looked for identity matches to proteins associated with toxicity or termed "toxins". In addition, literature searches for toxicity and for allergy to the species names were negative.

5.0 Conclusions

In using the criteria recommended by the Codex Alimentarius Commission for food safety (2003 and 2009), I find that there are no added risks from the consumption of this MnP protein considering our current understanding of allergens or possible cross-reactivity for allergy or any role in toxicity or anti-nutrient activity.

6.0 References

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7.0 Appendix: Allergenonline.org database, version 21 14 February 2021 (43 pages)

These pages can be submitted if requested

Infinite Enzymes/GreenLab Inc. Response to FDA questions for NPC 000019

Question 1: There is not a history of safe human consumption of the source organism, *Phanerochaete chrysosporium*. Are there other mushrooms that contain similar MnP enzymes with similar functions but are also consumed by humans, such as white button mushroom, shiitake mushroom? Such examples may provide support for the history of safe consumption of similar MnP enzymes in food.

Several publications document MnP activity in edible mushroom species (Ezeh, et al, 2019; Yehia, 2014; Hock et al, 2020; Xu, et al. 2012). These species include *Pleurotus ostreatus* (an oyster mushroom), *Agaricus bisporus* (a type of common button mushroom), *Lentinula edodes* (the shiitake mushroom), *Stropharia rugoso* (the "burgundy" mushroom), *Tricholoma lobyense* (gold good fortune mushroom), *Agrocybe cylindracea* (poplar mushroom), *Grifola frondosa* (hen-of-the-woods), *Pleurotus eryngii* (the largest of the oyster mushrooms) and others. Clearly a number of edible mushroom species produce MnP at some level and have been consumed safely for millennia. Taken together with other information and data in this submission, these findings support the conclusion that MnP enzymes are safe to consume.

Ezeh, O.J., et al 2019. Manganese Peroxidase (MnP) Activity of some Nigerian Edible Mushrooms. The Bioscientist Vol. 7 (1): 28-43.

Hock, O.G., et al 2020. Evaluation of the plastic degradation ability of edible mushroom species based on their growth and manganese peroxidase activity. Current Topics in Toxicology 16: 65-72.

Xu, J.Z., et al 2012. The relationship between lignin peroxidase and manganese peroxidase production capacities and cultivation periods of mushrooms. *Microbial Biotechnology,* 6, 241-247.

Yehia, R.S. 2014. Aflatoxin detoxification by manganese peroxidase purified from *Pleurotus ostreatus*. Brazilian Journal of Microbiology. 45, 1, 127-133.

Question 2: Please comment on the level of *P. chrysosporium* MnP expression in corn in quantitative terms. For example, one study (Clough et al., 2006) reported corn lines producing MnP at levels greater than 14% of total soluble protein or 500 mg/kg in certain cultivars. Is the level of MnP expression expected to have any impact/relevance to safety if there were inadvertently low levels in the commodity corn? It may be helpful to include consideration of the acreage currently grown of your corn and total corn acreage in the US and the uses of corn in food.

When Infinite Enzymes (now GreenLab) puts enzymes into corn through *A. tumefaciens* transformation, the first recovered seed are screened for the highest levels of expression in that generation. The corn that is used for transformation is typically a poor field variety, and the new high expressing lines must be backcrossed into elite inbred lines (2) that, when crossed with each other, produce a high yielding hybrid. During this backcross program, higher expression can be achieved and selected to yield much higher expression at the end of this activity. Increases in expression range from 10-50-fold. In the case of MnP, the final expression level is approximately 0.4% of dry weight of the kernels. This translates into ~4 g of MnP per kg of grain.

The acreage of corn grown yearly in the USA ranges from 80-100 million acres (USDA/NASS). Our current production field is 25 acres, or 0.0004% of the corn crop. GreenLab anticipates that within 10 years, production could be as much as 5000 acres, or 0.08% of the crop, again

generating limited opportunity for inadvertent presence in the food supply.

These points, along with the known safe consumption of MnP in some foods, support the conclusion that a low-level inadvertent presence of MnP in commodity corn would be safe.

Question 3: Please state whether the MnP enzyme is being expressed in field, sweet, and/or popping corn. Would the enzymatic reaction product of MnP be safe if the whole corn grains or processed corn products are consumed by humans? MnP catalyzes the oxidation of Mn(II) to Mn(III). If humans are exposed to the corn overexpressing MnP and the integrity of such corn grain is compromised and the cellular contents are released (e.g., through mastication or pulverization of the corn grain), can the released contents including MnP cause any harm to humans by generating reactive molecules (e.g., Mn (III)) in the buccal cavity (before MnP reaches the stomach and is digested)? Please provide a discussion of the basis for your conclusion.

The corn varieties used for this work are exclusively field corn. Regarding the possible production of reactive molecules in the buccal cavity, given the generally low levels of manganese and peroxides found in food, and the low likelihood of MnP-producing corn being found in food, the likelihood of producing potentially toxic levels of Mn(III) would be insignificant. These points, along with the known safe consumption of MnP in some foods, support the conclusion that a low-level inadvertent presence of MnP in commodity corn would be safe.

Question 4: (Figure 1 in Appendix II) Many extra bands are visible besides MnP protein in the gel. No method was provided to explain how MnP was purified. Please provide the purification method and the purity of the MnP protein. Please provide an explanation for the extra bands appearing in the gel.

Purification protocol: The protein is extracted from ground corn flour with 50 mM sodium tartrate, pH 4.5. GreenLab (formerly Infinite Enzymes Inc.) has shown that this is the best extraction method for avoiding many corn proteins and recovering >65% of the recombinant protein. Filter aid is added to the slurry at forty percent original weight of cornmeal, and the slurry is then filtered through a Whatman #1 filter paper in a Buchner funnel using a vacuum pump. The filtrate is precipitated with 95% ammonium sulfate overnight at 4^{o}C. The cake is added to 0.05M sodium tartrate, pH 4.5 buffer (~1 mL buffer/1 g "cake") and mixed for approximately 30 minutes. The cake slurry is filtered as noted above. In this case, protein is in the filtrate liquid. The filtrate is concentrated and desalted using ultrafiltration. Desalted fractions are applied to a Giga-Cap S-650M column and flow-through collected, which contains the MnP. Protein and MnP assays are performed on fractions. Appropriate fractions are combined. Purification is approximately 90%. Extra bands are background corn proteins (usually smaller bands) or multiples of MnP or glycosylated forms of MnP. The antibody recognizes some of the extra proteins on the western blot when the blot is highly overloaded, but not when lesser amounts of the protein are present. Thus, they are likely background proteins from corn that were in the protein mix that was used for rabbit injections for antibody preparation.

Question 5: There is an inconsistency in the size of MnP protein. Calculated molecular weight of the coding sequence (mature protein-292 AA) of MnP protein is 31 kDa (Page 37). In this submission, at page 21, section 4.1, the developer mentioned that Coomassie stained gel showed

MnP protein appeared as two primary bands (at 34 and 39 kDa) (Figure 1). However, the antibody binding was specific to a 37 kDa band (Figure 5). In a published article (Clough et al, 2006), an antibody detected a 46 kDa band.
Please clarify this inconsistency.

In both stained gel images (Figures 1 and 4 of study REG-IE in SGF&SIF 2021) there are three primary bands visible. One below 37 kDa that appears most intense, one at more than 37 kDa, possibly at 40 kDa and a lighter one at ~ 52 kDa. For antibody binding (Figure 5), a broad smeared band in MnP conc and MnP@2 as well as MnP half is recognized by the antibody at just below 37 kDa, and some other lighter bands are visible below 25 kDa and about 50 kDa (these are likely corn protein contaminants in the antigen injection). The more dilute sample loading, MnP low, shows more clearly a single band just below 37 kDa. That would likely be the primary protein MnP. We did not attempt to isolate the protein band and have any direct determination of sequence (e.g. LC-MSMS). The antibody binding was taken as affirmation of the protein identity in our study. Each gel system that is used to determine protein size has limitations and variations. In the original publication (Clough et al, 2006) it seems likely that the MnP was glycosylated, accounting for the larger than calculated size. Moreover, different gel systems appear to allow this protein to migrate at multiple molecular weights.

Question 6: The study report by Dr. Goodman noted that the *in vitro* digestion assay of MnP (Figure 2) was based on Astwood et al., 1996; Bannon et al., 2002; Thomas et al., 2004 (page 20, section 3 of **A**ppendix II). In the description of the degradation assay, the concentration of the substrate (MnP) and enzyme (pepsin) were not provided. The concentration of digestive enzymes added in the reaction mixture with respect to the concentration of MnP will affect the dynamics of the reaction and the degradation kinetics of MnP. Please provide the enzyme:substrate ratio used in the reaction mixture.
Please note that the complete citation of Astwood et al, 1996 was not provided in Section 6.0 References of Dr. Goodman's report.

The concentration of enzyme and substrate is not independently listed, except that the concentration of protein in the MnP sample was determined by 2-D Quant for stock at 14.04 mg/mol using BSA as a reference. The 100% target of protein in the wells, is based on the ratio of protein in the digest, with 1.47 micrograms loaded in each well, if 100% of target digest was there. The concentration of pepsin was tested and verified to fit within the expected activity of 10,000 units of pepsin activity per mg of test protein as described by the Ofori-Anti paper (2008) and the Thomas et al, paper (2004), which are referenced in the report. The activity of pepsin was verified based on digestion of bovine hemoglobin with amount measured by concentration of validation protein at 280 nm under fixed conditions as described in Ofori-Anti et al., 2004.

The complete citation for Astwood, et al 1996 is here:
Astwood, J, Leach, JN, and Fuchs, RL. 1996. Stability of food allergens to digestion in vitro. Nature Biotechnology 14: 1269–1273.

Question 7: Barley Alpha amylase was used as a signal peptide to localize the expression of MnP. Barley is a well-known source for allergenic proteins. In this submission, bioinformatics analyses did not include the amino acid sequence of Barley Alpha amylase for allergenicity assessment. Please justify its exclusion from the analysis or repeat the bioinformatic analysis using the MnP protein as expressed with the signal peptide.

Barley grain does include a few allergenic proteins that are listed in the WHO/IUIS Allergen Nomenclature database (www.allergen.org). Hor v 5 is a 30 kDa protein, Hor v 12 is profilin, Hor v 15 is an alpha-amylase inhibitor, Hor v 16 is alpha-amylase, Hor v 17 is beta-amylase and Hor v 20 is gamma-hordein 3. A few of those are included in the www.AllergenOnline.org database. The whole amino acid sequence listed by the developer was tested by FASTA and BLASTP against AllergenOnline.org and against the NCBI Protein database. The amino acid sequence did NOT match alpha-amylase of barley, nor did it match any other known allergens. Nevertheless, the alpha amylase signal sequence is not a part of any of the allergenic proteins from barley and should not be considered a potential allergenic sequence. Infinite Enzymes/GreenLab therefore concludes that there is no increased allergenic potential risk from inclusion of this MnP in its corn.

Question 8: Did Infinite Enzymes make any modifications to the amino acid sequence in comparison to the white rot fungus MnP protein sequence? If the amino acid sequence of the corn-expressed MnP has been modified, please provide the modified sequences and your rationale for concluding these modifications do not affect your safety assessment of MnP.

The original gene was synthesized with maize codon usage bias but none of the amino acids was changed.

Question 9: Post-translational modification (PTM) is one of the common events that happens during recombinant protein expression. PTM (glycosylation, phosphorylation, prenylation etc.) is required for protein folding, solubility, and stability in some systems. Glycosylation is a common PTM and it is also associated with allergenic food proteins. Is it known whether corn-expressed MnP is post- translationally modified? Would you expect glycosylated MnP or any modified form of MnP due to the PTM to have an increased potential to cross-react with known allergens? Please provide a discussion and analytical results, if available.

Using the periodic acid Schiff stain, laboratory personnel detected glycosylation of the enzyme, MnP. This glycosylation is not likely to be different than the glycosylation on other plant-derived proteins. In fact, other seed storage proteins from maize, such as embryo localized globulins, are glycosylated and have been consumed without allergic responses for many decades (Shewry and Halford, 2002). Thus, it is not likely that the MnP in the same embryo and expressed at the same temporal developmental stage would have a different pattern and contribute to allergenicity.

Shewry, PR, and NG Halford. 2002. Cereal seed storage proteins: structures, properties and role in grain utilization, Journal of Experimental Botany, 53:947-958.
https://doi.org/10.1093/jexbot/53.370.947

Question 10: On page 9, the Infinite Enzymes concluded that 'there are no added risks from the consumption of this protein considering our current understanding of allergens or possible cross- reactivity for allergy or any role in toxicity or anti-nutrient activity'. While your bioinformatic search terms include 'allergy' and 'toxicity', it did not include the term 'anti-nutrient'. On what basis did Infinite Enzymes conclude that MnP does not have a role in 'anti-nutrient' activity? Was it based on literature search? Please clarify.

Some compounds in food that are often categorized as "anti-nutrients" include lectins, oxalates, goitrogens, phytoestrogens, phytates, and tannins (Petroski, W and Minich, DM. 2020). A literature search for manganese peroxidase (MnP) and anti-nutrient was conducted and no notations were found that included MnP in any of the typical categories.

Infinite Enzymes/GreenLab Inc. Response to FDA questions for NPC 000019

Petroski, W and Minich, DM. 2020. Is There Such a Thing as "Anti-Nutrients"? A Narrative Review of Perceived Problematic Plant Compounds. Nutrients, 12: 2929.

Questions from CVM:

Q1: The developer states on page 8 that a literature search using the anamorph fungus name, *Sporotrichum pruinosum*, revealed a number of publications that included the term "toxin" or "toxic". The developer does not provide the citations for these scientific articles, and it is unclear if these references address animal safety. However, also on page 8, the developer states that the amino acid sequence was searched against a "toxin/toxic" subset of the NCBI Protein database and no sequences were identified that matched the manganese-dependent peroxidase to toxins/toxic subset of the NCBI Protein database. The developer should clarify this discrepancy.

In the publications noted, the presence of "toxin" or "toxic" in the text of the documents did not refer to toxicity of the organism (*S. pruinosum* or *P. chrysosporium*) but typically was a reference to myco**toxin** or some other notation in the publication to **toxic**ity unrelated to the organism itself. Hence no findings of toxin or toxicity of MnP in the NCBI Protein database would be expected.

Q2: The amino acid sequence of the manganese-dependent peroxidase was compared to the amino acid sequences of proteins suspected to have toxic or toxin like activity, using NCBI Protein and Entrez databases. The developer does not state what E-score cutoff was used for similarity of the manganese-dependent peroxidase protein with toxins in its database, and that information should be provided. The highest score that was include in the "toxic" search was 2.6 E-9.

There was no cutoff for E scores in the search for matches to toxins. *E*-score values will change depending on the length and complexity of the amino acid in matched proteins. The E score is a statistical estimate of the likelihood the protein match is meaningful in terms of likely evolutionary similarity. The number of sequences in the database searched also has a great influence over the score. The toxin search was performed using the whole NCBI protein database with limits on sequences selected based on keyword limits of "toxin" or "toxic". The smaller the E score, the higher the likelihood the sequences are sufficiently similar to believe they are likely to share overall sequence, structure and function. A BLASTP against the NCBI protein database without using key word matched sequences, shows 100 proteins from 100% identity match down to 67% identity match over the full length of proteins, with an E score of 1e-155 (that is 10-155...a very small number). Those are all manganese peroxidase like proteins. You cannot do a public NCBI Protein BLAST using key word limits since 2019. So, we ran it by having all protein sequences from NCBI Protein loaded on the University of Nebraska supercomputer, and then selecting NCBI Protein Accession numbers from NCBI using keyword limits of "toxin" and "toxic" to make lists for limited searches by BLAST on the super computer. When searched for "toxin" and "toxic" in the NCBI Protein list in September 2022, the number of proteins was:
"toxin" = it is 11,764,481sequences and for "toxic" = it is 1,471,534 sequences. That is out of a total number of sequences of 503,074,986 sequences. Those are 2.3% and 0.29% of all sequences in NCBI Protein, very large numbers of sequences. The question may seem hard to interpret. In essence, this MnP sequence, which is 292 amino acids long, would have a good possibility of meaningful homology to a toxin if the E-score is smaller than 1e-100, and have identity matches as great as 85%. Small snake venom proteins can have identity matches of >85% identity and E scores of 1e-34 to larger 4e-31 for 85% identity. But some other proteins in the top 100 alignments in NCBI protein can be 42% identity and 3e-4. They may or may not have related toxicity over their short (<75 AA) sequences and in fact it is usually the highly identical sequences that truly

share similar toxicities (Munawar A, Ali SA, Akrem A, Betzel C. 2018, Toxins 10:474).

There are no all-encompassing toxin databases, thus making searches for toxicity an artform that requires interpretation and the use of overall BLASTP analysis of proteins of interest and control proteins.

Q3: The developer does not provide any information on the specificity of the enzyme, especially for substances that are normally present in corn germ. The developer's website indicate that the proposed manganese-dependent peroxidase can be used for lignin degradation (primarily for cellulosic biofuel industry). Additionally, the developer states that the Mn3+ ions generated by manganese peroxidase acts as diffusible charge transfer mediators allowing the oxidation of several phenolic substrates, such as amines and phenolic lignin compounds. Some feedstuffs and ingredients used in animal food, such as the corn plant and corn silage, can contain some of the substrates targeted by the proposed manganese-dependent peroxidase. The developer should address if the presence of manganese-dependent peroxidase could affect the nutrient content of complete diets and the ensiling process of corn silage in the likelihood that the genetically engineered corn expressing the proposed manganese peroxidase was unintendedly included in animal food.

Manganese peroxidase uses manganese and hydrogen peroxide as substrates for activity. Because the corn seed does not contain peroxide, the enzyme has no activity. Also, manganese is generally not present in large amounts in any of the locations mentioned, including food. The corn kernel does not contain lignin, and thus the MnP is unlikely to have any effect on the quality of the feed.

The MnP enzyme is unlikely to be present in any silage, as the corn containing the enzyme will be grown to maturity and the seed harvested for enzyme extraction. Ensiled corn is harvested green and kept in the field for incubation and will never see an elevator or silo where grain is kept. If MnP is present in animal feed, it should be beneficial as it would participate in degrading lignin to make any cellulose more accessible to rumen microbes for digestion for energy in the feed (cellulosic feed, not grain).

Q4: The developer cites publications that indicate that MnP under specific experimental conditions mitigates mycotoxin presence. We note that mycotoxin mitigation in animal food is a different intended use than "detoxification and bioremediation" as proposed by the developer. Does the developer intend to use its manganese-dependent peroxidase to mitigate mycotoxins in/on grain, forage, byproducts or any other components to be used in animal food?

Greenlab (formerly Infinite Enzymes) has no current plans to use its manganese-dependent peroxidase corn products to mitigate mycotoxins in/on grain, forage, byproducts or any other components to be used in animal food.

Summary:

In total, Infinite Enzymes (GreenLab) has analyzed a number of factors to assess the risks of adverse impacts from the inadvertent presence of its manganese-dependent peroxidase producing corn on food and feed. Bioinformatic and analytical/testing assessments have been made for toxicity and allergenicity and no issues have been identified. Literature searches have noted that MnP has been consumed safely in foods for many years and no adverse impacts from

consumption in food or feed have have been noted for MnP. Additionally, given the processes to be used for growing, harvesting, and handling, the likelihood that this corn will make its way into commodity corn, even at a low-level presence, for food or feed is extremely low. Taken together, these points support the conclusion that the MnP protein is unlikely to cause allergic reactions in humans or be a toxin in humans or animals.

Infinite Enzymes/ GreenLab Inc. Response to CVM for NPC 000019

Questions from CVM (re: email from Dr. Kathleen Jones dated 10/27/2022):

1. Infinite Enzymes states on page 8 of its original submission that "the accession number lists for toxins (10,329 sequences) and toxic (9872 sequences) were loaded into the Holland Computing Center server at the University of Nebraska. However, in your amendment you state that for toxin 11,764,481 sequences and for toxic 1,471,534 sequences were identified and searched using the University of Nebraska supercomputer. Please clarify the large difference between the number of sequences inputed into the database that was searched.

Response:

In IE's (GreenLab's) initial submission, the original search parameters to identify reasonable sequence homology to toxins had already been narrowed to the lower number of sequences noted. IE noted this in its submission as looking for "…high sequence identity and small E scores…" The response to CVM's specific question looked broader at the terms "toxin" or "toxic" and therefore identified a much larger number of sequences. However, in no case was any toxic sequence found in the MnP.

2. Infinite Enzymes notes that the MnP sequence is 292 amino acids and discusses that E-score takes into consideration the length of the matched sequence and percent identity within this sequence. The bioinformatics analysis should address whether sequences within the 292 amino acid MnP sequence align with substances that are known to be toxins. It is unclear in your amendment whether you are reporting that there was a high E-score match between MnP sequence and small snake venom proteins and, if so, whether this would raise a potential safety concern. It is also unclear whether sequences within the proteins with lower E-score values (described as 42% identity and 3e-4) match to corresponding sequences within the toxin database. Please clarify.

Response:

The differences with the number of proteins identified using simple keyword matches "toxin" or "toxic" shows the complexity of identifying possible toxins. I do not think the differences in numbers should be the focus of question as we used different keyword limits to identify possibly interesting proteins. The last comparison was a maximum of sequences that might be considered. It was not intended to say that over 1 million sequences are likely toxins. Basically, there is a misunderstanding I think, as I was stating earlier that the there are no clear toxin databases and therefore, we used a general open-ended search for possible sequences of "toxin" or "toxic-like proteins". The numbers are very high, greater than 1.4 million for "toxic" and > 11.7 million for "toxin". Based on best sequence alignments, there appear to be some significant matches, but those are based on general overall alignments that do not indicate high sequence identity and functionality to "toxic" proteins. The E scores are influenced by overall length, and sequence complexity. Very small E scores can indicate conservation with identity matches of 85% and E scores of 1x10-34, whereas proteins of > 100 AA or > 200 AA can have lower identity scores that do not suggest shared functionality with even smaller E scores (1x 10 to the -90 or so) and no shared functionality. Thus, the bioinformatics matches do not realistically share toxicity as a function. Instead, it can be simply part of the molecules are

modestly conserved, for instance, in receptor binding. The large number of sequences identified by the simple keyword search for toxic or toxicity markedly over-predicts potential functionality. The overall sequence structure of the MnP proteins has been highly conserved over evolutionary history, but not focused on toxicity. The discussion about snake venom was simply an example to put E scores and lengths of alignment in perspective. There is NO relationship of snake venom and MnP proteins. We're sorry to have caused that confusion. Based on our knowledge of biology and overall protein structure, there should not be a concern that this protein is a likely toxin.

3. Greenlab (formerly Infinite Enzymes) states in its amendment that it has no current plans to use its manganese-dependent peroxidase corn products to mitigate mycotoxins in/on grain, forage, byproducts or any other components to be used in animal food. Should Greenlab change its intended uses to include use of this enzyme for specific intended uses in animal food in the United States, we recommend that Greenlab contact CVM's Division of Animal Food Ingredients to discuss regulatory and safety considerations as they pertain to the Federal Food Drug and Cosmetic Act that would need to be addressed prior to marketing and distribution of its manganese-dependent peroxidase corn products.

Response:

Greenlab fully understands the limitations of using its products for the uses described in its submitted documents. If Greenlab intends to extend the possible uses of its products beyond the current request, it will be sure to contact the appropriate entities within FDA, CVM, and other appropriate regulatory agencies.

GreenLab, Inc. Response to FDA regarding NPC 000019

Date: February 27, 2023

CFSAN has follow-up scientific questions for NPC 000019. We encourage you to ensure that your response is well-written, contains scientific data and information addressing our questions, and includes appropriate published literature citations.

1. *Please provide more substantive/technical information to support the similarity of MnP derived from the source organism, Phanerochaete chrysosporium, to MnP identified in edible mushroom species. Please consider providing detailed scientific/bioinformatic evidence that establishes these MnP isoforms are sufficiently similar to better support the history of safe consumption of similar MnP enzymes in food. Please feel free to cite additional references to support that the described level of similarity supports bridging of relevant safety information and history of safe consumption.*

Several publications document MnP activity in edible mushroom species (Ezeh, et al, 2019; Yehia, 2014; Hock et al, 2020; Xu, et al. 2012). These species include *Pleurotus ostreatus* (an oyster mushroom), *Agaricus bisporus* (a type of common button mushroom), *Lentinula edodes* (the shiitake mushroom), *Stropharia rugoso* (the "burgundy" mushroom), *Tricholoma lobyense* (gold good fortune mushroom), *Agrocybe cylindracea* (poplar mushroom), *Grifola frondosa* (hen-of-the-woods), *Pleurotus eryngii* (the largest of the oyster mushrooms) and others. MnP has also be found in the juice of *Luffa acutangula* (Rai, et al., 2017), an edible cucurbit also known as Chinese okra.

Additionally, GreenLab conducted a BLASTn search using the known MnP sequence (J04980.1) to assess sequence similarity with other organisms that would support a finding of safe consumption. The vast majority of organisms with sequence similarity were found to be other basidiomycetes, most of which are not generally consumed by humans as they are considered crust and/or white rot saprophytic fungi. Two noted organisms with sequence similarity that have food uses are noted as *Aspergillus niger* (79.26% nucleotide identity), which has been used for decades in food and industrial chemicals production (Cairns, et al 2018), and *Lentinula edodes* (66.63-67.42% nucleotide identity), the widely consumed Shiitake mushroom. Clearly there are food products that produce MnP and have been consumed safely for millennia.

References

Ezeh, O.J., et al 2019. Manganese Peroxidase (MnP) Activity of some Nigerian Edible Mushrooms. The Bioscientist Vol. 7 (1): 28-43.

Yehia, R.S. 2014. Aflatoxin detoxification by manganese peroxidase purified from *Pleurotus ostreatus.* Brazilian Journal of Microbiology. 45, 1, 127-133.

Hock, O.G., et al 2020. Evaluation of the plastic degradation ability of edible mushroom species based on their growth and manganese peroxidase activity. Current Topics in Toxicology 16: 65-72.

Xu, J.Z., et al 2012. The relationship between lignin peroxidase and manganese peroxidase production capacities and cultivation periods of mushrooms. *Microbial Biotechnology,* 6, 241-247.

N. Rai, M. Yadav* and H.S. Yadav. 2017. Manganese Peroxidase from *Luffa acutangula* Fruit Juice. Curr Biochem Engineering. Vol 44: 188 – 193.

Cairns, TC, C Nai, and V Meyer. 2018. How a fungus shapes biotechnology: 100 years of *Aspergillus niger* research. Fungal Biol and Biotechnol. 5, 13. https://doi.org/10.1186/s40694-018-0054-5

2. *Please describe if the subject MnP is heat-labile or if it the pH/conditions of the buccal cavity would impact MnP enzymatic activity, and if this supports the safety of low-level inadvertent presence of MnP in the food supply.*

Reports vary slightly on the optimum temperature and pH conditions for activity of manganese peroxidase from *Phanerochaete chrysosporium*. Generally, however, the optimum activity temperatures are noted as ~30-40 °C and pH conditions of 4.5 (Urek and Pazarlioglu, 2007; Cuoto et al., 2006). Activity levels are demonstrated to drop significantly, however, between an optimum pH of 4.5 and pH 5.5 and between temperatures of 37 °C and 45 °C (Urek and Pazarlioglu, 2007). A recent review of a wide range of MnPs from many organisms expands these ranges somewhat with optimum temperature activities noted as 30-70 °C and pH optima between 3.0 and 5.5 (Kumar and Arora 2022).

Given the known pH of the buccal cavity as ~6.2-7.6 (Baliga, et al 2013), the activity of MnP from *Phanerochaete chrysosporium* in this environment is expected to be quite low. MnP requires peroxide for activity and the buccal cavity has little or no peroxide (Ryan and Kleinberg, 1995). Additionally, while chelated Mn(III), a product of MnP, is stable in soils and alkaline aqueous solutions (Schuisky et al., 1999), Mn(III) as a free hydrated ion is not stable in aqueous solution such as in food or the mouth (Greenwood and Earnshaw, 1984). Finally, given the short time in the buccal cavity, along with the requirements for substrates Mn(II), H^+ and H_2O_2, significant activity of any MnP that might be contained in a low level presence situation with this corn, the formation of any potentially toxic compounds within the mouth would be highly unlikely.

References

Urek, RO, NK Pazarlioglu. 2007. Enhanced production of manganese peroxidase by *Phanerochaete chrysosporium*. Braz Arch Biol Technol 50(6): 913-920.

Couto, S.R., Moldes, D. and Sanromán, M.A. 2006. Optimum stability conditions of pH and temperature for ligninase and manganese-dependent peroxidase from *Phanerochaete chrysosporium*. Application to *in vitro* decolorization of Poly R-478 by MnP. *World J Microbiol Biotechnol* **22**, 607–612. https://doi.org/10.1007/s11274-005-9078-0

Kumar A and Arora PK (2022). Biotechnological Applications of Manganese Peroxidases for Sustainable Management. Front. Environ. Sci. 10:875157. doi: 10.3389/fenvs.2022.875157

Baliga, S., S Muglikar, and R Kale. 2013. Salivary pH: A diagnostic biomarker. J Indian Soc Periodontol. Vol 17: 461–465. doi: 10.4103/0972-124X.118317

Ryan, CS and I Kleinberg. 1995. Bacteria in human mouths involved in the production and utilization of hydrogen peroxide. Arch. In Oral Biol. Vol 40: 753-763.

Schuisky, P, A-S Ullstrom, and I Persson. 1999. Long-term Stability of Manganese (III) Complexes in Aqueous Soil Slurries. Acta Agric. Scand., Sect. B, Soil and Plant Sci. 49:39-43. DOI: 10.1080/09064719950135687

Greenwood, NN and A Earnshaw. 1984. The Chemistry of the Elements. Pergamon Press, Oxford. 1228 p

3. *We request that the notifier addresses the following:*
    a. *Is the enzymatic activity of the enzyme in corn retained after common food processing conditions, i.e., in cooked corn or processed corn-based foods as consumed by humans?*

GreenLab conducted enzyme activity assays at increasing temperatures with its MnP purified from corn. At a concentration of 0.1 mg/ml MnP, no enzyme activity was detected after 5 minutes at 50 or 55 °C while 75% activity was retained at 5 minutes at 45 °C. No activity of MnP was found at 10 minutes at 45 °C. As such, processing involving even moderate heating for any length of time would be expected to entirely inactivate the MnP enzyme in this corn. Other reports cited previously also support the idea that MnP from *P. chrysosporium* loses activity at high temperatures (i.e., Cuoto et al., 2006; Kumar and Arora 2022).

References

Couto, S.R., Moldes, D. and Sanromán, M.A. 2006. Optimum stability conditions of pH and temperature for ligninase and manganese-dependent peroxidase from *Phanerochaete chrysosporium*. Application to *in vitro* decolorization of Poly R-478 by MnP. *World J Microbiol Biotechnol* **22**, 607–612. https://doi.org/10.1007/s11274-005-9078-0

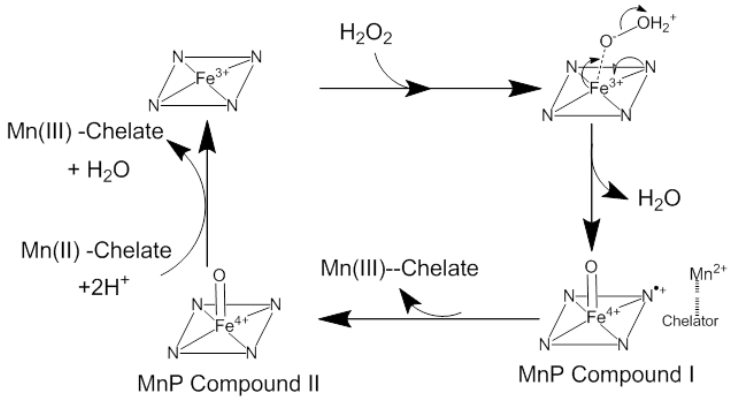
Kumar A and Arora PK (2022). Biotechnological Applications of Manganese Peroxidases for Sustainable Management. Front. Environ. Sci. 10:875157. doi: 10.3389/fenvs.2022.875157

    b. Once processed by commercial or home-cooking methods, would the MnP in the MnP corn be inactivated and/or removed (e.g. corn oil, starch)?

While extremely small traces of protein may be found in corn oil or starch, as noted previously, increased temperatures that are likely to be used during typical food processing, either at home or commercially, would be expected to entirely inactivate the MnP in this corn.

4. *As we mentioned at the meeting (between FDA and Infinite Enzymes on August 4, 2022), please provide information about any known secondary enzyme activities of the MnP enzyme.*

In addition to the required substrates Mn(II), H^+ and H_2O_2, MnP requires one cofactor, heme, and the presence of Ca^{2+} for activity. And as such, there are other possible chemical reactions that can and do occur under various conditions. Mn(III) is one possible product but, as previously noted, it is quite unstable in aqueous solution and it is typically rapidly chelated when produced. A more complete picture of the chemistry of MnP redox reactions is shown here:



(Rai, et al., 2017)

References

N. Rai, M. Yadav* and H.S. Yadav. 2017. Manganese Peroxidase from *Luffa acutangula* Fruit Juice. Curr Biochem Engineering. Vol 44: 188 – 193.