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Characterization and safety evaluation of HPPD W336, a modified 4-hydroxyphenylpyruvate dioxygenase protein, and the impact of its expression on plant metabolism in herbicide-tolerant MST-FGØ72-2 soybean

Rozemarijn Dreesen[a,*], Annabelle Capt[b], Regina Oberdoerfer[c], Isabelle Coats[d], Kenneth, Edward Pallett[a]

[a] Bayer CropScience N.V. – Innovation Center, Tech Lane Ghent Science Park 38, B-9052, Gent, Belgium
[b] Bayer S.A.S., Bayer CropScience, 355 rue Dostoïevski, 06903, Sophia Antipolis, France
[c] Bayer A.G., CropScience Division, Alfred-Nobel-Straße 50, 40789, Monheim, Germany
[d] Bayer CropScience L.P., 2 T.W. Alexander Drive, Research Triangle Park, NC, 27709, USA

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ABSTRACT

By transgenic expression technology, a modified 4-hydroxyphenylpyruvate dioxygenase enzyme (HPPD W336) originating from *Pseudomonas fluorescens* is expressed in MST-FGØ72-2 soybean to confer tolerance to 4-benzoyl isoxazole and triketone type of herbicides. Characterization and safety assessment of HPPD W336 were performed. No relevant sequence homologies were found with known allergens or toxins. Although sequence identity to known toxins showed identity to HPPD proteins annotated as hemolysins, the absence of hemolytic activity of HPPD W336 was demonstrated *in vitro*. HPPD W336 degrades rapidly in simulated gastric fluid. The absence of toxicity and hemolytic potential of HPPD W336 was confirmed by *in vivo* studies. The substrate spectrum of HPPD W336 was compared with wild type HPPD proteins, demonstrating that its expression is unlikely to induce any metabolic shifts in soybean. The potential effect of expression of HPPD W336 on metabolic pathways related to tyrosine was investigated by comparing seed composition of MST-FGØ72-2 soybean with non-genetically modified varieties, demonstrating that expression of HPPD W336 does not change aromatic amino acid, homogentisate and tocochromanol levels. In conclusion, HPPD W336 was demonstrated to be as safe as other food proteins. No adverse metabolic effects were identified related to HPPD W336 expression in MST-FGØ72-2 soybean.

1. Introduction

Genetically modified (GM) crops which are tolerant to the non-selective herbicides glyphosate and glufosinate have proven their value for modern agriculture (Green, 2014). However, current issues with weed resistance and weed population shifts show that today's growers would benefit hugely if they could use different herbicide treatments to avoid over reliance on a single herbicide (Green, 2018). Hence, the development of alternative weed control technologies has become essential. This publication introduces a modified protein to generate a novel generation of herbicide-tolerant GM crops.

4-Hydroxyphenylpyruvate dioxygenase (HPPD; EC 1.13.11.27, EC 1.14.2.2) catalyzes the oxidative decarboxylation of 4-hydroxyphenylpyruvate (4-HPP) to form homogentisate (HGA). HPPD is a ubiquitous enzyme present in virtually all aerobic organisms and it performs a key step in the catabolism of tyrosine which results in the formation of fumarate and acetoacetate (Fig. 1).

In plants, HGA also has a key anabolic role as a precursor in the biosynthesis of tocochromanols (tocopherols and tocotrienols) and prenylquinones, such as plastoquinone. The former have an essential anti-oxidant function, whereas plastoquinone is an essential cofactor for phytoene desaturase, a key enzyme in the biosynthesis of carotenoid pigments (Norris et al., 1995). In addition, plastoquinone is a key component of the photosynthetic electron transport chain in photosystem II. Inhibition of HPPD has severe consequences for the plant (Fig. 1), causing phototoxicity because the reduction of plastoquinone levels leads to impaired electron transport. Furthermore, the reduction of phytoene desaturase activity prevents carotenoid synthesis, essential for chloroplast development and photosynthesis (Pallett et al., 1998). The impact of inhibition of HPPD leads to a 'bleached' phenotype of

* Corresponding author.
E-mail addresses: rozemarijn.dreesen@bayer.com (R. Dreesen), annabelle.capt@bayer.com (A. Capt), regina.oberdoerfer@bayer.com (R. Oberdoerfer), isabelle.coats@bayer.com (I. Coats), ken.pallett@blueyonder.co.uk (K.E. Pallett).

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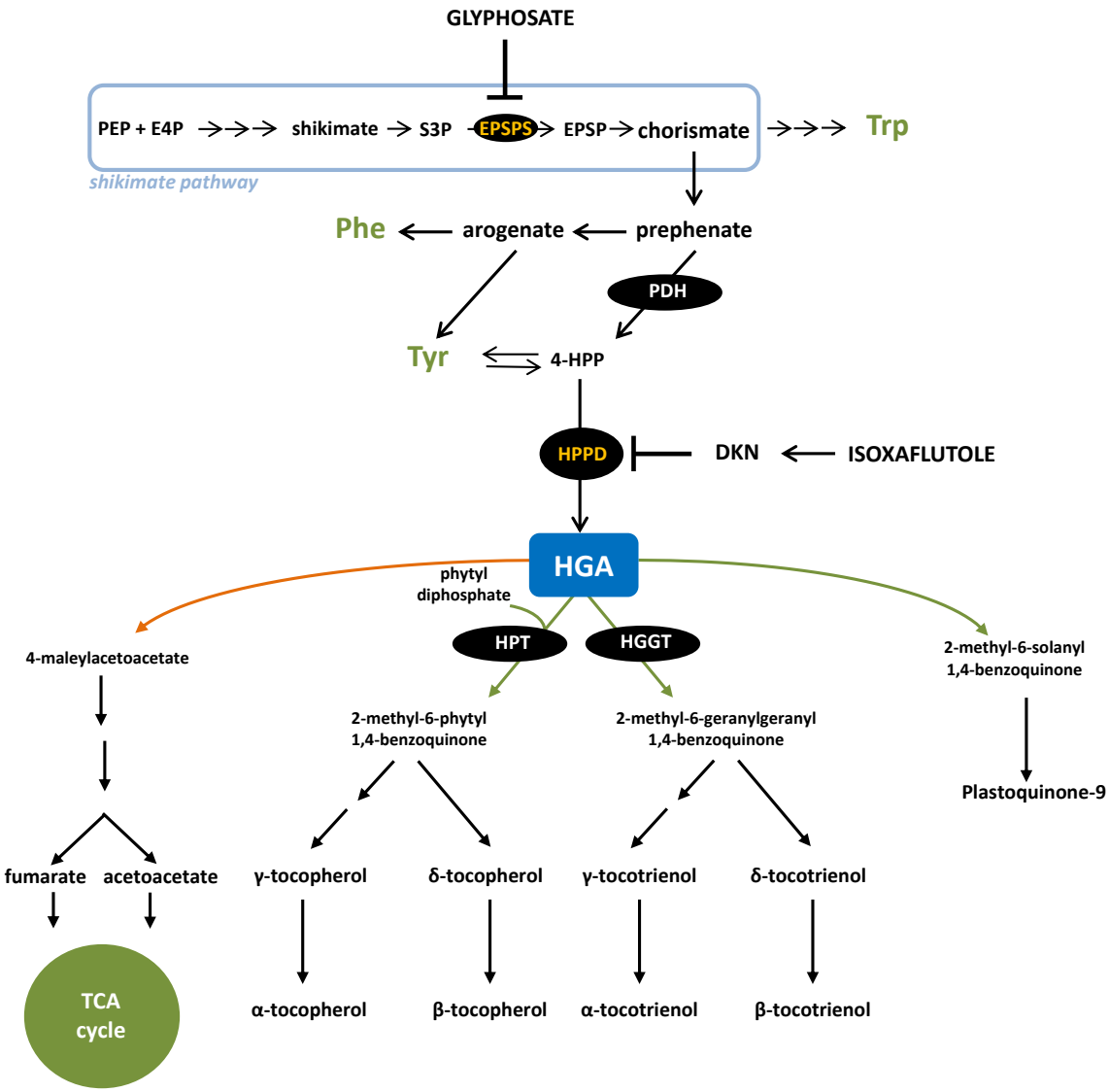

Fig. 1. Biosynthesis of aromatic amino acids and degradation of tyrosine in plants related to the anabolic and catabolic function of homogentisate, the reaction product of HPPD enzymatic activity.
Chorismate is the precursor of the aromatic amino acids tyrosine (Tyr), phenylalanine (Phe) and Tryptophan (Trp) and is the resulting compound from the shikimate pathway. One of the key enzymes of this pathway is 5-enoyl-pyruvylshikimate 3-phosphate synthase (EPSPS), which is the target of the herbicide glyphosate. Tyrosine is degraded into homogentisate (HGA) in two enzymatic steps, of which the second step is catalyzed by 4-hydroxyphenylpyruvate dioxygenase (HPPD), the target of the herbicide isoxaflutole. HGA is a central compound in the degradation pathway of tyrosine since it is a substrate of many enzymes. Within the catabolic part of the pathway (indicated by the orange arrow) HGA is degraded into fumarate and acetoacetate, which are ultimately recycled within the tricarboxylic acid (TCA) cycle. The anabolic pathways starting from HGA are indicated by the green arrows. The action of the enzymes in this anabolic pathway lead to the formation of tocopherols, tocotrienols and plastoquinone-9.
Legend: 4-HPP: 4-hydroxyphenylpyruvate; DKN: diketonitrile; E4P: D-erythrose 4-phosphate; HPT: homogentisate prenyl transferase; HGGT: homogentisate geranyl geranyl transferase; PEP: phosphoenolpyruvate; PDH: prephenate dehydrogenase; S3P: shikimate-3-phosphate.

susceptible species, because of the dual effects of reduced pigment biosynthesis and impaired chloroplast development (Fig. 2, panel A2). The anabolic role of HPPD in plants makes this enzyme a highly effective target site for herbicides such as 4-benzoyl isoxazoles, *e.g.* isoxaflutole and the triketones, *e.g.* mesotrione[1] (Luscombe et al., 1993; Mitchell et al., 2001), and the expression of modified HPPD in plants has led to herbicide tolerance (Matringe et al., 2005; Clarke et al., 2013; Kramer et al., 2014; Siehl et al., 2014).

MST-FGØ72-2 is a GM soybean line developed jointly by Bayer CropScience and MS Technologies, expressing the HPPD W336 protein, which has a single amino acid (AA) substitution at position 336 and is derived from the *Pseudomonas fluorescens* strain A32 *hppd* gene (Genbank Acc N° AAE74448). When treated with isoxaflutole, MST-FGØ72-2 soybean retains the normal 'green' phenotype (Fig. 2, panel B2). The replacement of the glycine residue at position 336 by a tryptophan leads to reduced sensitivity to diketonitrile (DKN), which is the herbicidal principle of isoxaflutole (Pallett et al., 1998; Matringe et al., 2005). The point mutation has a strong effect on DKN sensitivity but has a moderate impact on the normal enzyme kinetics of HPPD (Matringe et al., 2005). In addition to isoxaflutole, MST-FGØ72-2 soybean also shows tolerance to the herbicide glyphosate due to the expression of a modified 5-enoyl-pyruvylshikimate 3-phosphate synthase (2mEPSPS) derived from maize. The 2mEPSPS protein was previously demonstrated as being safe for food and feed consumption (Herouet-Guicheney et al., 2009).

[1] The use of these types of herbicides is pending regulatory approval.

Fig. 2. Phenotype of MST-FGØ72-2 soybean compared with non-GM soybean variety MST39 upon spraying with isoxaflutole under greenhouse conditions.
The non-GM soybean variety MST39 was not treated (panel A1, control) and treated with a herbicide containing isoxaflutole as an active ingredient (ai) which blocks HPPD action (panel A2), respectively. MST-FGØ72-2 soybean was not treated (panel B1, control) and treated with isoxaflutole (panel B2), respectively. In plants, HGA is the reaction product of HPPD enzymatic activity with 4-HPP as a substrate. HGA is a precursor in the biosynthesis of tocochromanols and prenylquinones. Consequently, the impact of inhibition of HPPD by isoxaflutole leads to a 'bleached' phenotype of susceptible plants, because of the dual effects of reduced pigment biosynthesis and impaired chloroplast development. In MST-FGØ72-2 soybean, HPPD W336 is expressed, which is less sensitive to isoxaflutole. This is demonstrated by the same phenotype in panel B2 as the control in panel B1. Observation was made 20 days after treatment.

The objective of this paper is to describe the characterization and the safety assessment of HPPD W336 which was conducted as part of the regulatory approval process for MST-FGØ72-2 soybean. This assessment follows the internationally recognized approach for assessing the safety of newly expressed proteins based on a weight of evidence approach (Codex Alimentarius Commission (C.A.C.), 2009; EFSA, 2011; Delaney et al., 2008; Herouet-Guicheney et al., 2009; Raybould et al., 2013).

In addition, it was verified whether the modification of HPPD W336 has any impact on its substrate specificity. Furthermore, the levels of aromatic amino acids, homogentisate and tocochromanols in MST-FGØ72-2 soybean and the non-GM counterpart were compared to assess the potential impact of the expression of HPPD W336 on the tyrosine degradation pathway and the metabolism of HGA.

2. Materials and methods

2.1. Purification of HPPD proteins from Escherichia coli

Large-scale batches of *Escherichia coli* (*E. coli*)-purified HPPD W336 were produced for characterization and safety assessment (HPPD W336-1 to -3). HPPD W336 consists of 358 amino acids and was previously designed by site directed mutagenesis of the sequence encoding wild type HPPD from *P. fluorescens* strain A32 at position 336 (Matringe et al., 2005). The HPPD W336 coding sequence was ligated into either expression vector pSE420 (Life Technologies, Carlsbad, CA) or pET09 (Merck Millipore, Darmstadt, Germany) and expressed in either *E. coli* strain MG1655 or BL21 (Life Technologies, Carlsbad, CA). Starting from a 50 mL seed culture in Terrific Broth containing 100 μg/mL Kanamycin A, *E. coli* cells were ultimately grown by high density fermentation of 10 L medium containing 7.5 g/L of KH_2PO_4, 2.46 g/L $MgSO_4.7H_2O$, 53.71 g/L D-glucose, trace elements and 0.5 g/L Antifoam P2000 at a constant pH of ~7.5 and constant supply of D-glucose after depletion. Expression was induced by isopropyl-1-thio-β-galactopyranoside (IPTG) at an OD_{600} of ~60–80. Fermentation was stopped after 48 h. Harvested *E. coli* cells were disrupted by a high pressure homogenizer. The cell lysate was clarified by centrifugation, after which DNA and RNA were precipitated using 1% streptomycine sulphate. The supernatant was subjected to different rounds of ammonium sulphate (AMS) precipitation to a final saturation of 45–50%. Pellets were dissolved in 20 mM HEPES pH 7.5 containing 10 μM of either ferrous or ferric chloride and precipitated by a final concentration of ~7% hydroxyapatite. Resulting pellets were further purified by ion-exchange chromatography over a Q-Sepharose column (GE Healthcare, Piscataway, NJ). Batch HPPD W336-3 was further purified using hydrophobic interaction chromatography over Toyopearl Phenyl 650 C resin (Tosoh Bioscience LLC, Tokyo, Japan). Most pure eluted fractions, as judged by SDS-PAGE, were pooled and concentrated using dialysis tubing (Amicon®, Merck Millipore Darmstadt, Germany) with a molecular weight cut-off of 12–14 kDa to a buffer containing 5 μM of either ferrous or ferric chloride. The resulting protein batches were aliquoted and lyophilized. Before each use, protein was reconstituted in 50 mM Tris-HCl-containing buffer (pH 7.5). The purity of the lyophilized bacterially-produced HPPD W336 batches was determined by SDS-PAGE and is in the range of 96–99% HPPD W336 over total protein content (Table 1 of Dreesen et al., submitted).

To determine substrate specificity, small-scale batches of HPPD W336, the wild type, non-modified HPPD from *P. fluorescens* strain A32 (Genbank Acc N° AAE74448) and native soybean HPPD (Genbank Acc N° ABU25694) were purified from *E. coli*. The corresponding HPPD coding sequences were ligated into expression vector pSE420 (Life Technologies, Carlsbad, CA) and expressed in *E. coli* strain MG1655.

Cells were grown at 37 °C in 5 L of Terrific Broth-DRY High Nutrient Growth Medium (MO BIO Laboratories, Carlsbad, CA) containing 100 µg/mL of Carbenicillin. At an OD_{600} of ~0.5, the cells were induced by the addition of IPTG, further cultivated for 16 h at 18 °C and harvested by centrifugation. Pellets were resuspended in 10 mM potassium phosphate (pH 7.5), 1 mM DTT and 1 mM benzamidine and disrupted using an IMA-Disintegrator S glass-bead disintegrator. The resulting homogenate was cleared by centrifugation. DNA and RNA were precipitated using 1% streptomycine sulphate. The supernatant was subjected to AMS precipitation at 50% saturation followed by centrifugation. The pellet was resuspended in 12 mL of 50 mM potassium phosphate buffer pH 7.5, 1 mM DTT (buffer A) and desalted. Further purification of HPPD proteins occurred on a 45 mL Q-Sepharose Fast Flow column (GE Healthcare, Piscataway, NJ). Elution of the column occurred using a 450 mL linear gradient of 0–1 M KCl in buffer A. For each HPPD protein, the purest fraction (≥90%), as judged by SDS-PAGE, was retained.

2.2. Purification of HPPD W336 protein from MST-FGØ72-2 soybean leaves

MST-FGØ72-2 soybean was produced by direct transfer of a chimeric construct to wild type soybean which contained a gene cassette in which the HPPD W336 encoding sequence (Matringe et al., 2005) is fused with an optimized transit peptide to target the protein into the chloroplast (Herouet-Guicheney et al., 2009). This transit peptide is cleaved off during maturation of HPPD W336.

HPPD W336 was purified from MST-FGØ72-2 soybean under two different sets of conditions by affinity chromatography. The protein preparation from the first purification method was used for physico-chemical characterization while the second protein preparation was performed under more gentle physiological conditions to preserve and test for functionality. Affinity columns were prepared by covalent attachment of crude anti-HPPD W336 rabbit polyclonal serum (Agdia, Elkhart, IN) to AminoLink Plus Immobilization resin (pH 10.0) according to the manufacturer's instructions (Thermo Fischer Scientific, Oberhausen, Germany). Each time, MST-FGØ72-2 soybean leaves were ground in a pre-chilled Waring blender, under the continuous addition of fresh dry ice. For the first purification method, 150 mL of PBST buffer (Agdia Inc., Elkhart, IN) containing cOmplete™ protease inhibitor (Roche, Basel, Switzerland) was added to 50 g of ground leaf sample, after which the mixture was incubated for 30 min at 4 °C under continuous stirring. The extract was clarified by centrifugation and vacuum filtering. The affinity column was pre-conditioned with IgG elution buffer (Thermo Scientific, Rockford, IL) followed by PBS buffer (Thermo Scientific, Rockford, IL). After addition of clarified extract, unbound proteins were washed from the column with PBS buffer after which HPPD W336 protein was eluted by IgG elution buffer. The pH of the eluent was buffered to approx. pH 7.5, concentrated and exchanged into PBS buffer using Amicon® centrifugal filter devices (Merck Millipore Darmstadt, Germany). To purify functional HPPD W336 protein, 333 mL of Tris buffer containing 50 mM Tris (pH 7.5), 100 mM KCl, 1 mM $FeCl_2$, 1 mM $FeCl_3$, 5% glycerol and cOmplete™ protease inhibitor was added to 100 g of ground plant leaves. The resulting mixture was rotary rocked for 30 min at 5 °C, filtered through Miracloth (Calbiochem, San Diego, CA) and centrifuged to clarify. The affinity column was conditioned with 50 mM ethanolamine (pH 9.5) followed by PBS. After addition of clarified extract, the column was washed twice with Tris buffer. HPPD W336 protein was eluted from the column with 25 mL of 50 mM ethanolamine pH 9.5, after which the pH of the eluent was reduced to approx. pH 7.5, concentrated and exchanged into PBS buffer using Amicon® Ultra centrifugal filter devices.

2.3. Physico-chemical and functional characterization of MST-FGØ72-2 soybean-purified and bacterially-produced HPPD W336

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed to verify the identity of MST-FGØ72-2 soybean-purified and bacterially-produced HPPD W336 (HPPD W336-1) by tryptic digest mapping (Aebersold, 1993; Billeci and Stults, 1993). Further peptide fragmentation analysis by MALDI MS/MS was performed on 15 peptides.

The N-terminal amino acid sequence was determined by means of Edman degradation (Hunkapiller and Hood, 1983). For both proteins, strong signals were obtained enabling reliable determination of residues for 7 subsequent cycles.

The apparent molecular mass of the bacterially-produced and MST-FGØ72-2 soybean-purified HPPD W336 proteins was determined by densitometry analysis of a Coomassie-stained SDS-PAGE gel. Western blot analysis was performed to assess immuno-reactivity against a polyclonal anti-HPPD W336 antibody (Agdia, Elkhart, IN, provided by MS Technologies).

Enzymatic activity of the bacterially-produced and MST-FGØ72-2 soybean-purified HPPD W336 proteins was assessed by an assay based on the determination of unconsumed 4-HPP substrate in a mixture containing HPPD W336 protein sample, catalase, and ascorbate after 1 h incubation period at room temperature. Remaining 4-HPP was derivatised with 2,4-dinitrophenylhydrazine (DNPH) to 2,4-dinitrophenylhydrazone. Afterwards, NaOH was added to terminate the reaction. The formed amber color was measured photometrically at 405 nm (E_{405}). Enzyme activity was estimated by calculation of a proportional value of E_{405} absorbances observed for the assay lacking enzyme with HPP (negative control), enzyme containing assays with 4-HPP (sample) and enzyme containing assay without 4-HPP (blank):

$$100 \times \frac{[E405(negative\ control) - E405(sample)]}{[E405(negative\ control) - E405(blank)]}$$

Consequently, assessment of functionality of the HPPD W336 protein samples was limited to a qualitative scoring in which samples were accepted to show HPPD functional activity if the calculated proportional value was ≥10%. A non-GM counterpart derived protein preparation, which endured the same purification procedure as MST-FGØ72-2 soybean-purified HPPD W336 was included as a negative control to demonstrate specificity of the assay.

2.4. HPPD W336 protein sequence comparison with known allergens and known toxins

The amino acid sequence of HPPD W336 was compared with all sequences present in the Comprehensive Protein Allergen Resource (COMPARE) public allergen database managed by the International Life Sciences Institute's Health and Environmental Sciences Institute (www.comparedatabase.org; version 1, released on February 3, 2017). To assess potential toxicity, the HPPD W336 sequence was compared to all sequences present in the public NCBI non-redundant protein database (version 2015.0117, released on Jan 17, 2015).

The FASTA program was used for alignment (version 35.04 from 2009; Pearson and Lipman, 1988). The scoring matrix was BLOSUM50 and default settings were used for gaps opening and extension penalties (*i.e.* -10/-2). For the search against the COMPARE database, an E-value threshold of 10 was used. For the search against the NCBI non-redundant protein database, an E-value threshold of 0.1 was used. The relevance of the matches was assessed by examining the alignment parameters (*e.g.* identity, similarity, length of alignment, presence of

gaps, E-value), as well as the published information on potential toxicity/allergenicity of the matching protein.

2.5. In vitro assessment of potential hemolytic activity of the HPPD W336 protein

Whole blood from 3 male and 3 female healthy human donors was used for the evaluation of hemolysis.

HPPD W336 batch HPPD W336-1 (purity 97%) was tested at 3 concentrations: 1, 10 and 100 µg/mL of whole blood. The highest concentration corresponds to the theoretical blood concentration after an intravenous injection in mice at 10 mg/kg of body weight, taken into account a blood volume of approximately 90 mL/kg of body weight (Riches et al., 1973). The other two dose levels (1 and 10 µg/mL) were included to evaluate any potential dose effect.

Saponin was used as a positive control at a concentration known to induce 100% hemolysis (3% w/v). Water at a 1:5 dilution in whole blood was used as an independent control as it induces a consistent and low level of hemolysis in all human donors. In addition, the alpha hemolysin from *Staphylococcus aureus* was included as a reference hemolysin (Sigma-Aldrich, Saint Quentin Fallavier, France), at a concentration close to that of the highest HPPD W336 concentration (114 µg/mL, corresponding to 5000 U/mL). 50 mM Tris HCl buffer (pH 7.5) was used as a negative control.

The HPPD W336, saponin and staphylococcal alpha hemolysin were prepared as 5x solutions in 50 mM Tris HCl pH 7.5. Test protein or controls in whole blood, as well as unprocessed whole blood, were incubated for 45 min, at approximately 37 °C, centrifuged for 10 min at 3000 rpm at approximately 20 °C, and processed to collect plasma supernatant. The optical density (OD) of the supernatants was measured at 540 nm in triplicate, by a microplate reader. The hemolysis was considered as biologically significant when OD values were at least two-fold higher than the negative control (*i.e.* 50 mM Tris-HCl pH 7.5) or unprocessed plasma, whichever was the lowest.

2.6. Pepsin resistance of the HPPD W336 protein in human simulated gastric fluid

The experimental design for the pepsin resistance test was based on Thomas et al. (2004). HPPD W336 batch HPPD W336-2 (2.5 mg/mL) was added to the human simulated gastric fluid (SGF; 2 mg/mL NaCl, pH 1.2) in the presence of pepsin (10 U/µg of HPPD W336 protein; Sigma-Aldrich, Saint Quentin Fallavier, France). The protein was incubated in SGF at 37 °C. At various time points samples were taken and the reaction was stopped by adding $NaHCO_3$ pH 11.0 (52 mM final concentration).

Appropriate controls included HPPD W336 without pepsin, pepsin alone, and a 10% loading control (to prove the detection of at least 10% of the original protein quantity). Reference proteins horseradish peroxidase (HRP) and ovalbumin (OVA), known to be digested rapidly and slowly, respectively, were tested in parallel to verify the activity of the pepsin. The samples were analyzed by SDS-PAGE and western blot analysis using a specific rabbit polyclonal anti-HPPD W336 antibody (Agdia, Elkhart, IN; provided by MS Technologies).

2.7. In vivo toxicity assessment of the HPPD W336 protein

Mice were obtained from Charles River Laboratories (St. Germain-sur-l'Arbresle, France) and acclimatized to laboratory conditions for at least 6 days prior to treatment. Diet and water were supplied *ad libitum* during the study, except during fasting. Before each study, the animals were randomly assigned to groups using an automatic procedure (either XMS Path/Tox Version 4.2.2 or Pristima, version 6.3.2 build 17, Xybion Corp.), which ensured a similar body weight distribution among groups.

2.7.1. Acute oral toxicity study in the mouse

HPPD W336 was evaluated for acute oral toxicity in female Crl:OF1 mice. This study was conducted based on the OECD TG n° 420 (2001) and on the US EPA OCSPP Guideline n° 870.1100 (2002) and in accordance with the animal welfare guidelines.

HPPD W336 batch HPPD W336-1 was formulated as a homogenous suspension at 50 mg/mL in 50 mM Tris-HCl, pH 7.5. The animals were 8 weeks old on the day of treatment. After overnight fasting, 5 female Crl:OF1 mice were administered HPPD W336 by oral gavage at the limit dose level of 2000 mg/kg body weight (2 administrations of 20 mL/kg body weight separated by 3 h). The control group received bovine serum albumin (BSA) protein in the same vehicle at a similar dose.

All animals were observed for mortality and clinical signs at least once during the first 30 min after each dosing, periodically during the first 24 h post dosing, and every day thereafter for 15 days. Body weights were measured weekly.

At final sacrifice on study day 15, after overnight fasting, all animals were weighed (terminal body weight), anesthetized, then exsanguinated under a deep anesthesia before necropsy. The necropsy included macroscopic examination of the abdominal and thoracic cavities, major organs and tissues. Brain, liver, spleen and kidney were weighed fresh at scheduled sacrifice and sampled. These samples were preserved in 10% neutral buffered formalin for possible histological examination. Spleen was processed and embedded in paraffin wax. Histological slides were prepared for all animals and stained with hematoxylin and eosin and subjected to histopathological examination.

The statistical analyses were performed using XMS Path/Tox (Version 4.2.2). Briefly, the homogeneity of variance was tested using an F-test. If $p > 0.05$, a 2-sided *t*-test was used. If $p \leq 0.05$ in the F-test, the analysis depended on the parameters. For terminal body weight and organ weights, the data were analyzed using a modified 2-sided *t*-test. For body weight and food consumption, the same procedure was repeated after log transformation of the data.

2.7.2. 28-day repeated oral toxicity study in the mouse

HPPD W336 was evaluated for sub-acute oral toxicity in male and female C57BL/6J mice. This study was conducted based on the OECD TG n° 407 (2008) and the EFSA Guidance for risk assessment of food and feed from genetically modified plants (2011).

HPPD W336 batch HPPD W336-3 was formulated every 3 days as a homogenous suspension at 50 mg/mL in 50 mM Tris-HCl, 136 mM NaCl, 2.7 mM KCl, pH 7.4. The formulated protein was analyzed for concentration and homogeneity using the Bio-Rad DC Protein Assay (Bio-Rad, Marnes-la-Coquette, France). Stability of the protein in the formulation was verified for a period of 3 days.

The animals were 7 weeks old at the start of treatment. HPPD W336 was administered by gavage to C57BL/6J mice (10/sex/group), daily for 28 days at the dose of 1000 mg/kg/day (each administration at 20 mL/kg body weight). The control group received the vehicle alone.

The mice were observed daily for mortality and clinical signs. Body weight and food consumption were recorded weekly. A detailed physical examination was performed weekly. Ophthalmological examination was performed at study termination.

Blood samples were taken on study day 25 for hematology and on study day 29 for clinical chemistry. Hematology and clinical chemistry analysis were performed (parameters are listed in Table 3 and Table 4).

At final sacrifice on study day 29, after overnight fasting, all animals were weighed (terminal body weight), and sacrificed by exsanguination under deep anesthesia. The necropsy included macroscopic examination of the abdominal and thoracic cavities, major organs and tissues. A range of organs were weighed. All tissues and organs were sampled and preserved in 10% neutral buffered formalin (except eye, optic nerve, Harderian gland, epididymis and testis, which were fixed in Davidson's fixative) and embedded in paraffin wax (except the exorbital gland, larynx/pharynx and nasal cavities). Histological slides were stained

with hematoxylin and eosin. All slides were examined microscopically by a qualified pathologist and representative slides were peer-reviewed by a second pathologist.

The statistical analyses were performed using Pristima (version 6.3.2 build 17, Xybion Corp.). Briefly, the homogeneity of variance was tested using an F-test. If $p > 0.05$, a 2-sided *t*-test was used. If $p \leq 0.05$ in the F-test, the analysis depended on the parameters. For terminal body weight, organ weights, clinical chemistry parameters and a set of hematology parameters (hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, % neutrophils, % lymphocytes, % reticulocytes), the data were analyzed using a modified 2-sided *t*-test. For body weight, food consumption and the rest of hematology parameters (red blood cell count, platelet count, white blood cell count, neutrophil count, lymphocyte count, reticulocyte count), the same procedure was repeated after log transformation of the data.

2.8. Enzymatic substrate specificity of HPPD proteins

The enzymatic specificity of wild type *P. fluorescens* HPPD, HPPD W336 and native soybean HPPD towards a set of substrates was determined by two experimental approaches.

Firstly, specificity of *P. fluorescens* HPPD and HPPD W336 was determined towards the natural substrate 4-HPP. Herefore, appropriate amounts of purified HPPD protein were incubated in a solution containing 4-HPP, ascorbate and catalase. The amount of formed HGA was quantified using a HPLC system equipped with a C-18 column (Eurospher 100-5; Knauer, Berlin, Germany). K_m and V_{max}-values were determined by fitting initial velocities of HPP turnover at different 4-HPP concentrations to the Michaelis-Menten equation using Model 350 of the ID Business Solutions Ltd Xlfit version 5.1.0.0 software suite (Guildford, United Kingdom). K_{cat}-values were calculated from V_{max}-values.

Secondly, HPPD enzymatic activities were determined photometrically by the same principle as described above for the functional characterization of batch HPPD W336-1 and MST-FGØ72-2 soybean-purified HPPD W336. Substrates tested were 4-HPP, HGA, phenylpyruvate (PP), α-ketoisocaproate (KIC), and α-keto-γ-(methylthio)butyrate (KMTB) purchased from Sigma-Aldrich (Darmstadt, Germany), as well as 3,4-dihydroxyphenylpyruvate (3,4-dHPP; Fluorochem, Hadfield, UK). To determine 4-HPP conversion, a calibration curve was applied to determine 4-HPP concentrations at the onset of the assay and after an incubation time of 24 min. Three µg of HPPD enzyme was added to the reaction. Enzyme activity (U; nmol substrate consumed per min) was calculated based on the difference in E_{405} absorbances (ΔE_{405}) between assays lacking enzyme (negative control) and the enzyme containing assays using 4-HPP as a standard. For the determination of the alternative substrate specificity of the HPPD enzymes, the 4-HPP in the assay mixture was replaced by either PP, KIC, KMTB or 3,4-dHPP. The incubation time was doubled to 48 min and the amount of HPPD enzyme in the reaction was increased 7.5–10 times. Corresponding calibration curves were used for each of the alternative compounds. To evaluate conversion of alternative compounds by the HPPD enzymes, specific activities (U/mg HPPD protein) in relation to alternative substrates were expressed relatively to specific activities in relation to 4-HPP.

2.9. Compositional analysis of soybean grain

2.9.1. Material

Compositional analyses of soybean grain were performed on MST-FGØ72-2 soybean, non-GM soybean MST39, and six non-GM commercial varieties. In a first experiment, soybean seed samples from eight field trial sites in the USA, which were representative for commercial soybean production, were selected for compositional analyses. Each trial site included plots of three of the six commercial soybean varieties. Plots were replicated and arranged in a randomized complete block design (RCBD). Standard soybean production practices were applied. A subset of the plots with MST-FGØ72-2 soybean received one application of isoxaflutole (70 g active ingredient (ai)/hectare) during pre-emergence and one application of glyphosate (Roundup PowerMAX; 1032 g ai/hectare supplemented with 1426 g ai/hectare ammonium sulphate) at 4–5 Leaf post emergence growth stages. Seed samples were harvested at normal maturity and subsequently shipped to the composition laboratory where they were transferred to frozen storage (-20 ± 5 °C) until analysis.

From a second, independent field trial experiment, soybean grain were selected for analysis of the HGA content. This experiment was performed under a similar study design as described above. Differences between the trials are detailed in Table 3 of Dreesen et al. (submitted).

2.9.2. Analyses

The compositional analyses included the determination of nutrients and anti-nutrients including amino acids, tocopherols and tocotrienols. All analyses, except HGA determination, were performed by the Eurofins Scientific Nutrition Analysis Center (Des Moines, IA). HGA content determination was performed by Covance Laboratories, Inc. (Madison, WI). Prior to analysis, samples were homogenized to powder in a blender using liquid nitrogen. Commonly accepted, validated methods were used for sample analysis. (AOAC International, 2000; Aalberse, 2000; Barkholt and Jensen, 1989; Cort et al., 1983; Henderson and Books, 2010; McMurray et al., 1980; Scavu et al., 2005; Schuster, 1988; Speek et al., 1985).

With the exception of α-, δ- and γ-tocotrienol and HGA levels (which were below the Level of Quantification (LOQ)), all data were combined over all sites and subjected to analysis of variance (ANOVA) using a linear mixed model

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \delta k(j) + \varepsilon_{ijk}$$

where Y_{ijk} is the observed response for entry i at trial site j and block k, μ the overall mean, α_i the fixed effect associated with entry (*i.e.* the MST-FGØ72-2 soybean treated or untreated with herbicides, the non-transgenic counterpart, and the commercial soybean varieties, see Table 6), β_j the random effect associated with site, $\alpha\beta_{ij}$ the random effect for the interaction of entry by site, $\delta k(j)$ the random effect associated with block nested within site and ε_{ijk} the random error. Based on the mixed model, entry differences were estimated and presented with 95% confidence intervals, along with the p-values (*t*-test) for the entry differences. Differences were considered significant only when the *t*-test p-value was < 0.05. The statistical analysis was performed using SAS version 9.3 (WINDOWS 7).

3. Results

3.1. MST-FGØ72-2 soybean-purified HPPD W336 and bacterially-produced HPPD W336 are equivalent

Given the limited expression of the HPPD W336-encoding gene in MST-FGØ72-2 soybean, it is not feasible to extract the protein in sufficient amounts from this matrix for use in *in vitro* and *in vivo* safety studies. *E. coli*-produced HPPD W336 batches are acceptable as a surrogate protein when the structural and functional equivalence with the MST-FGØ72-2 soybean-purified HPPD W336 is demonstrated by means of a side-by-side comparison of different protein characteristics (Delaney et al., 2008; Raybould et al., 2013).

Physico-chemical properties were determined for both *E. coli*-produced HPPD W336 batch HPPD-W336-1 and MST-FGØ72-2 soybean-purified HPPD W336. The sequence identity of both the *E. coli*-produced batch HPPD W336-1 and MST-FGØ72-2 soybean-purified HPPD W336 was demonstrated using N-terminal sequencing and peptide mapping. The theoretical N-terminal sequence of the first eight amino

A

1 MADLYENPMG LMGFEFIEFA SPTPGTLEPI FEIMGFTKVA THRSKNVHLY

51 RQGEINLILN NEPNSIASYF AAEHGPSVCG MAFRVKDSQK AYNR**ALELGA**

101 **QPIHIDTGPM ELNLPAIKGI GGAPLYLIDR FGEGSSIYDI DFVYLEGVER**

151 **NPVGAGLKVI DHLTHNVYR**G RMVYWANFYE KLFNFREARY FDIKGEYTGL

201 TSKAMSAPDG MIRIPLNEES SKGAGQIEEF LMQFNGEGIQ HVAFLTDDLV

251 KTWDALKKIG MRFMTAPPDT YYEMLEGRLP DHGEPVDQLQ ARGILLDGSS

301 VEGDKRLLLQ IFSETLMGPV FFEFIQRKGD DGFGEWNFK**A** **LFESIERDQV**

351 **R**RGVLTAD

B

1 MADLYENPMG LMGFEFIEFA SPTPGTLEPI FEIMGFTKVA THRSKNVHLY

51 RQGEINLILN NEPNSIASYF AAEHGPSVCG MAFRVKDSQK AYNRALELGA

101 QPIHIDTGPM ELNLPAIK**GI** **GGAPLYLIDR** FGEGSSIYDI DFVYLEGVER

151 NPVGAGLK**VI** **DHLTHNVYRG** RMVYWANFYE KLFNFREARY FDIKGEYTGL

201 TSKAMSAPDG MIRIPLNEES SKGAGQIEEF LMQFNGEGIQ HVAFLTDDLV

251 KTWDALKKIG MRFMTAPPDT YYEMLEGRLP DHGEPVDQLQ AR**GILLDGSS**

301 **VEGDKR**LLLQ IFSETLMGPV FFEFIQR**KGD** **DGFGEWNFKA** **LFESIERDQV**

351 **R**RGVLTAD

Fig. 3. MALDI-TOF coverage of *E. coli*-produced and MST-FGØ72-2 soybean-purified HPPD W336 proteins.
The coding sequence of HPPD W336 was used to produce surrogate protein in *E. coli* and is expressed in MST-FGØ72-2 soybean. The HPPD W336 protein consists of 358 AA and differs from the non-modified HPPD protein from *P. fluorescens* by a glycine (G) to tryptophan (W) substitution at position 336 (Matringe et al., 2005) which is indicated by grey shading. MALDI-TOF coverage results of *E. coli*-produced HPPD W336 and MST-FGØ72-2 soybean-purified HPPD W336 protein are shown in panel A and panel B, respectively. Underlined regions correspond to tryptic peptides that were identified using MALDI-TOF MS, bold regions correspond to peptides identified by MALDI-TOF MS/MS.

acids of the HPPD W336 protein deduced from the DNA sequence of the gene used for expression in MST-FGØ72-2 soybean is MADLYENP. The N-terminal sequence obtained for both the *E. coli*- and MST-FGØ72-2 soybean-purified HPPD W336 is ADLYENP, hence lacking the predicted N-terminal methionine. A missing methionine is often observed in proteins expressed in prokaryotic and eukaryotic organisms (Bradshaw et al., 1998). Peptide mapping analysis showed that sequence coverage of the theoretical amino acid sequence of HPPD W336 by the tryptic peptides generated from the *E. coli*-produced HPPD W336 was 51.4%. For MST-FGØ72-2 soybean-purified HPPD W336, this was 46.4%. For each HPPD W336, four of the identified tryptic peptides were confirmed by sequencing. Taken together, the identity of both samples was confirmed (Fig. 3).

The apparent molecular mass determination of *E. coli*-produced HPPD W336 and HPPD W336 purified from MST-FGØ72-2 soybean leaves is presented in Fig. 4 (Panel A). The electrophoretic mobilities of both proteins were indistinguishable and corresponded to a molecular mass of ~37.2 kDa. Given the technical range of the applied method, this result is in line with the expected theoretical molecular mass calculated from the amino acid sequence (40.3 kDa). Immunoblot analysis of *E. coli*-produced HPPD W336 and HPPD W336 purified from MST-FGØ72-2 soybean leaves (Fig. 4, Panel B) shows that for both samples, a comparable band of the expected molecular mass was observed, demonstrating immuno-reactivity against an anti-HPPD W336 antibody.

In addition, it was demonstrated that both the *E. coli*-produced HPPD W336 and the HPPD W336 purified from MST-FGØ72-2 soybean leaves were not glycosylated (Fig. 3 of Dreesen et al., submitted).

By a qualitative enzymatic activity assay, significant relative conversion values of the 4-HPP substrate were observed for both the bacterially-produced HPPD W336 and the HPPD W336 purified from MST-FGØ72-2 soybean leaves. Consequently, both proteins possess HPPD-specific activity.

Independently, the characteristics of *E. coli*-produced HPPD W336 batches HPPD W336-2 and -3 were determined. Results obtained demonstrated their comparable physico-chemical properties with batch HPPD W336-1 as well as their HPPD functionality (Fig. 1, Fig. 2 and Table 1 of Dreesen et al., submitted).

Taken together, the results demonstrate that the *E. coli*-produced HPPD W336 is structurally and functionally equivalent to MST-FGØ72-2 soybean-purified HPPD W336, warranting the use of bacterially-produced HPPD W336 for *in vitro* and *in vivo* safety assessments.

3.2. HPPD W336 has no relevant sequence identity with known allergens

No relevant identities were found between the HPPD W336 sequence and known allergen sequences from the COMPARE database. The best scoring match had 24.1% identity (57.0% similarity) with an allergen (accession number CAA31575) over 79 amino acids, corresponding to an E-value of 2.6.

3.3. HPPD W336 has no hemolytic potential

As expected, HPPD W336 had sequence identities with other HPPD proteins (also called HPPDase; MelA; enzyme classification E.C.1.13.11.27) from various origins.

A few of these HPPD proteins are annotated as hemolysins (also called VllY; accession numbers WP_023493232, BAO82111, WP_022521422, EEW10892, and EEW05126). These hemolysin-annotated proteins share 55.0%–73.0% identity with the HPPD W336 protein.

The annotation of these HPPD proteins as hemolysins was due to indirect *in vitro* experimental data and was not due to a direct hemolytic activity of these proteins. The hemolytic phenotype was first described for the LlY protein from *Legionella pneumophila* (also called legiolysin), and the VllY protein from *Vibrio vulnificus* (Wintermeyer et al. 1994; Chang et al. 1997), when the expression in their source organisms or in *E. coli* led to the formation of brown pigment, associated with α-hemolysis of surrounding human and sheep red blood cells (Steinert et al. 2001). In these experiments, the formation of pigment was shown to be caused by the conversion of the tyrosine present in the culture medium

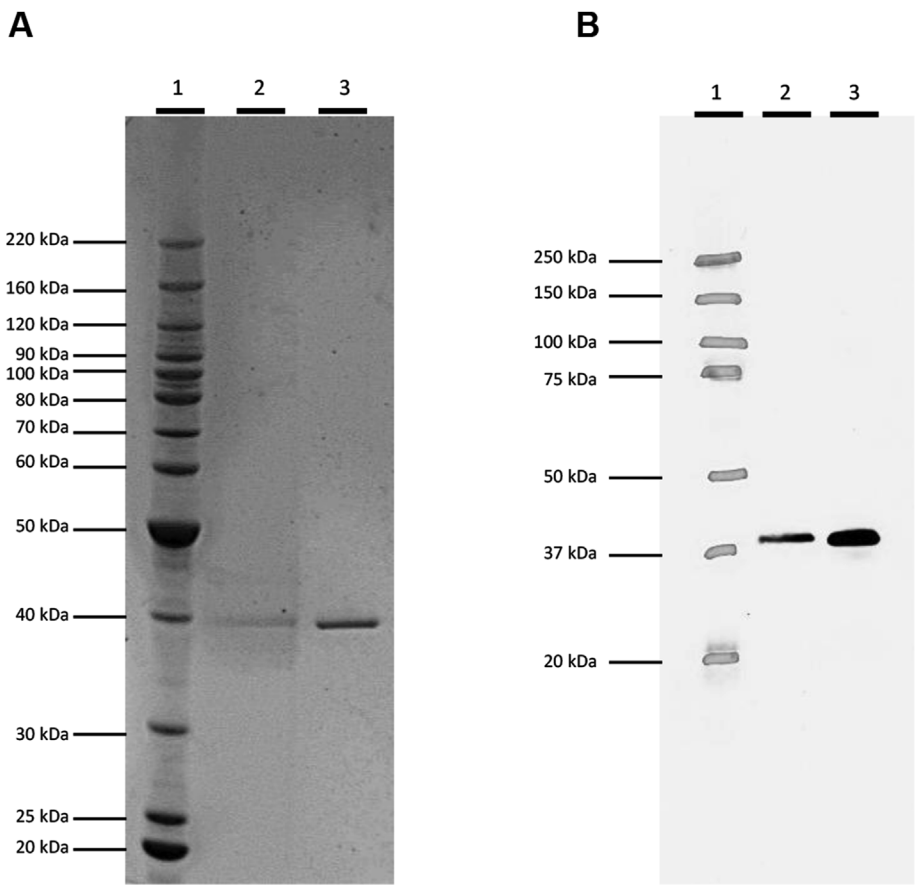

Fig. 4. SDS-PAGE (panel A) and western blot (panel B) analysis demonstrating the identity of the MST-FGØ72-2 soybean-purified and *E. coli*-produced HPPD W336 proteins.
Aliquots of MST-FGØ72-2 soybean-purified HPPD W336 and *E. coli*-produced HPPD W336 batch HPPD W336-1 were separated by means of SDS-PAGE using a 10% Bis-Tris gel and MOPS SDS running buffer. The gel was Coomassie-stained using Imperial Protein Stain (ThermoFisher Scientific). Lane 1: Molecular mass marker; Lane 2: ~400 ng of MST-FGØ72-2 soybean-purified HPPD W336 (amount loaded based on total protein content; approx. 50% HPPD W336/total protein); Lane 3: ~400 ng of *E. coli*-produced HPPD W336 batch HPPD W336-1 (97% HPPD W336/total protein).
For western blot analysis, SDS-PAGE was performed likewise, after which proteins were transferred to a PVDF membrane. The blot was probed using rabbit anti-HPPD W336 antibody and developed using an ECL system. Lane 1: Molecular mass marker; Lane 2: ~8 ng of MST-FGØ72-2 soybean-purified HPPD W336 (amount loaded based on total protein content); Lane 3: ~8 ng of *E. coli*-produced HPPD W336 batch HPPD W336-1.

into HGA, triggered by the HPPD activity of LlY or VllY. When released into the medium, HGA spontaneously underwent oxidation and polymerized into the hemolytic melanin-like pigment (Hegedus and Nayak, 1994; Steinert et al., 2001). It is thus reasonable to postulate that any other HPPD protein, *e.g.* from common foods with a history of safe consumption, would stimulate the production of HGA and consequently result in the same α-hemolytic phenotype under similar experimental conditions. Therefore, LlY and VllY do not have any direct hemolytic activity. All other matching proteins annotated as hemolysins (accession numbers WP_023493232, BAO82111, WP_022521422, EEW10892, and EEW05126) were annotated as such because of their sequence similarity with the LlY and VllY proteins, but their functions are putative.

Consequently, the identities between HPPD W336 and other HPPD proteins annotated as hemolysins are not indicative of a potential direct toxicity of HPPD W336.

To confirm the absence of any direct hemolytic activity of HPPD W336, an *in vitro* test was conducted. As expected, this test demonstrated no spontaneous hemolysis caused by HPPD W336, whereas the positive control saponin promoted maximal hemolysis (Fig. 5). The staphylococcal α-hemolysin induced strong hemolysis in samples from all six donors, which confirmed the ability of the test to detect a signal in the presence of a protein with a known hemolytic activity.

3.4. HPPD W366 is rapidly degraded in simulated gastric fluid

At time zero of incubation with SGF, the HPPD W336 band and the pepsin band at approx. 36 kDa were clearly visible (Fig. 6, panel A). At 30 s and all subsequent incubation times, there were no visible HPPD W336 bands or digestion fragments. As the 10% loading control was clearly visible, these results indicate that HPPD W336 was degraded at > 90% within 30 s. This result was confirmed in the western blot (Fig. 6, panel B).

As expected, the reference proteins HRP and OVA were rapidly and slowly digested (data not shown). HRP was completely digested within 30 s of incubation in the SGF, while OVA was visible up to 20 min of incubation.

In addition to the SGF test, an *in vitro* test in simulated intestinal fluid was performed, which demonstrated that HPPD W336 was completely digested by pancreatin within 30 s of incubation (Fig. 4 of Dreesen et al. submitted). HPPD W336 was also shown to lose its activity very rapidly when heated at 60 °C or more, indicating that the protein will be inactivated under conditions (*e.g.* heat treatment) commonly applied in the processing of soybeans for food and feed products (Fig. 5 of Dreesen et al. (submitted).).

3.5. The HPPD W336 protein shows no toxicity in an acute oral toxicity study in the mouse

No mortality or clinical signs were observed in any animals during the course of the study. No effect on body weight was observed in HPPD W336-treated animals, when compared with the control animals.

At final necropsy, there were no treatment-related macroscopic observations recorded. Mean absolute and relative spleen weights were statistically significantly lower in the HPPD W336 treated group when compared with the control group (Table 1). Histopathological evaluation of the spleen from all animals showed no treatment-related observations. Therefore, this change in spleen weight was not considered to be treatment-related due to the low magnitude of the change, the high inter-individual variability and the absence of association with any treatment-related macroscopic or microscopic findings.

Therefore, acute oral treatment with HPPD W336 at 2000 mg/kg body weight did not induce any evidence of systemic toxicity in the OF1 female mouse.

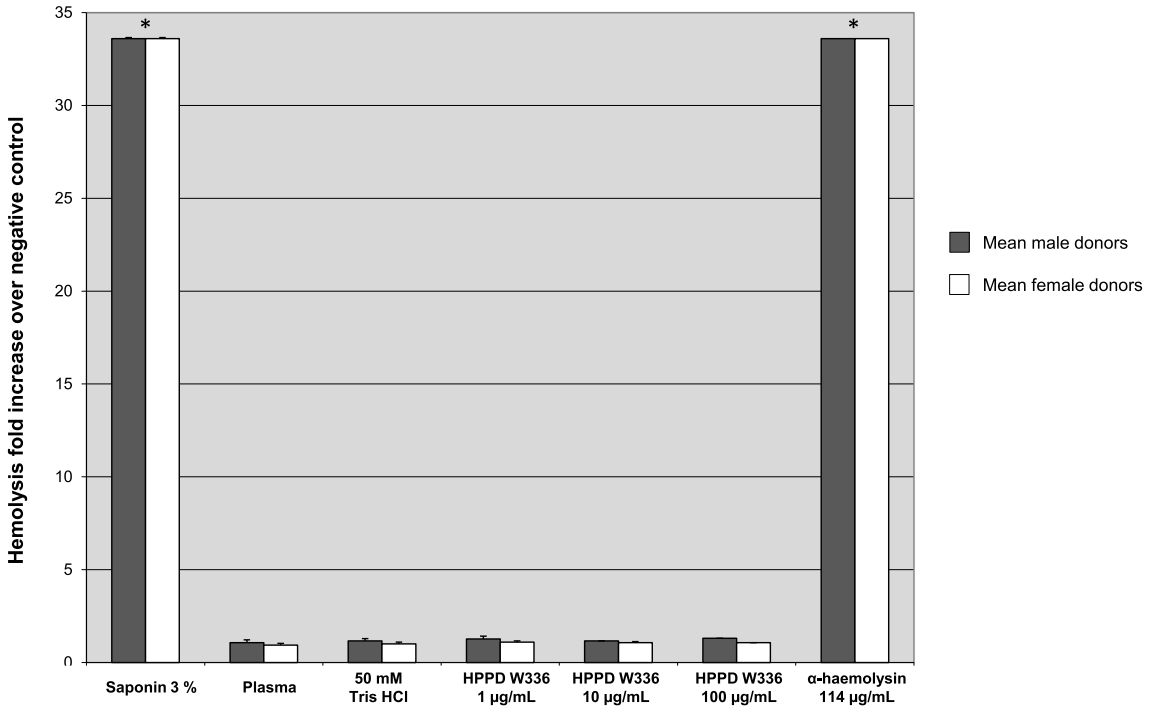

Fig. 5. *In vitro* hemolytic activity test on human blood.
Results represent the observed optic density (OD) relative to the OD from 50 mM Tris-HCl or unprocessed plasma, whichever was the lowest. Standard deviations are shown. * positive results (*i.e.* ≥ 2-fold increase).

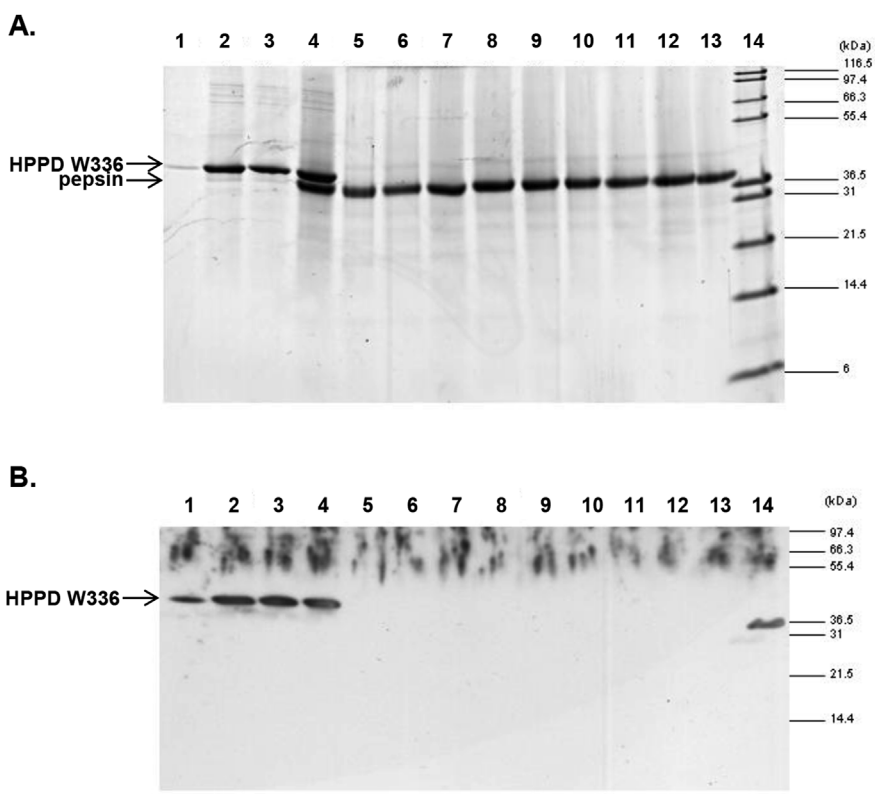

Fig. 6. Degradation of HPPD W336 in SGF.
Panel A: SDS-PAGE. After 10 min heating at > 90 °C with Laemmli solution, the samples were separated by means of SDS-PAGE using 10–20% Tris/Tricine gels and Tris/Tricine/SDS running buffer (Bio-Rad, France). The gel was stained using Coomassie blue (ThermoFisher Scientific, France). Lane 1: 10% loading control with HPPD W336 (~100 ng), time 0, no pepsin; Lane 2: HPPD W336, time 0, no pepsin (~1 µg); Lane 3: HPPD W336, time 60 min, no pepsin (~1 µg); Lane 4: HPPD W336, time 0 (~1 µg); Lane 5: HPPD W336, time 0.5 min; Lane 6: HPPD W336, time 2 min; Lane 7: HPPD W336, time 5 min; Lane 8: HPPD W336, time 10 min; Lane 9: HPPD W336, time 20 min; Lane 10: HPPD W336, time 30 min; Lane 11: HPPD W336, time 60 min; Lane 12: SGF alone, no protein, time 0; Lane 13: SGF alone, no protein, time 60 min; Lane 14: molecular weight marker.
Panel B: western blot. SDS-PAGE was performed likewise, with ~1000 fold less protein loaded on the gel. The gel was transferred to a PVDF membrane. The membrane was incubated in the presence of a specific rabbit polyclonal anti HPPD W336 protein antibody (Agdia, Ekhart, IN; provided by MS Technologies), and developed using an ECL detection system (GE Healthcare Life Sciences, France). Lane 1: 10% loading control with HPPD W336 (~100 pg), time 0, no pepsin; Lane 2: HPPD W336, time 0, no pepsin (~1 ng); Lane 3: HPPD W336, time 60 min, no pepsin (~1 ng); Lane 4: HPPD W336, time 0 (~1 ng); Lane 5: HPPD W336, time 0.5 min; Lane 6: HPPD W336, time 2 min; Lane 7: HPPD W336, time 5 min; Lane 8: HPPD W336, time 10 min; Lane 9: HPPD W336, time 20 min; Lane 10: HPPD W336, time 30 min; Lane 11: HPPD W336, time 60 min; Lane 12: SGF alone, no protein, time 0; Lane 13: SGF alone, no protein, time 60 min; Lane 14: molecular weight marker.

Table 1
Acute oral toxicity study: terminal body weight, absolute organ weights, organ to body weight ratio and organ to brain weight ratio.

Females	Unit	TBW	BRAIN			LIVER			SPLEEN			KIDNEY(S)		
		(g)	(g)	(% to bw)	(% to bn wt)	(g)	(% to bw)	(% to bn wt)	(g)	(% to bw)	(% to bn wt)	(g)	(% to bw)	(% to bn wt)
CONTROL GROUP	(n)	5	5	5	5	5	5	5	5	5	5	5	5	5
	Mean	22.6	0.44	1.938	100	0.98	4.346	225.306	0.094	0.4172	21.6116	0.32	1.414	73.164
	Std dev	0.5	0.02	0.116	0	0.09	0.322	26.307	0.006	0.0201	1.9502	0.03	0.119	6.929
HPPD W336 GROUP	(n)	5	5	5	5	5	5	5	5	5	5	5	5	5
	Mean	22.9	0.42	1.838	100	1	4.363	238.108	0.074[a]	0.3248[b]	17.701[a]	0.32	1.39	75.657
	Std dev	1.1	0.02	0.097	0	0.08	0.321	25.277	0.014	0.0568	3.257	0.04	0.169	8.716

TBW: Terminal body weight; % to bw: organ to body weight ratio; % to bn wt: organ to brain weight ratio; Std dev: standard deviation.
[a] The group mean is significantly different from the control at $p \leq 0.05$.
[b] The group mean is significantly different from the control at $p \leq 0.01$.

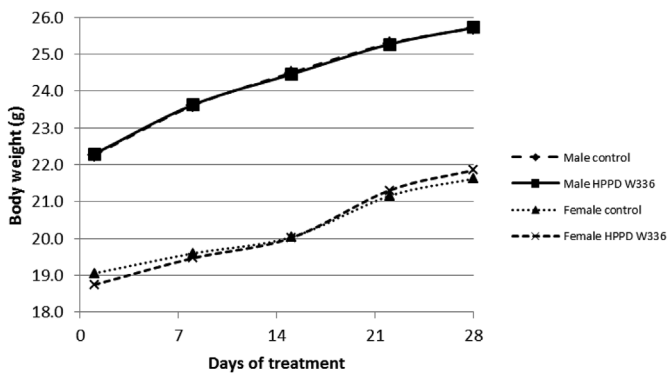

Fig. 7. Mean body weights in the 28-day mouse study.
Mean body weights of male and female mice from the HPPD W336-treated and control groups (N = 10). No statistical differences were detected in either sex.

3.6. HPPD W336 is not toxic in an oral 28-day repeated dose study in the mouse

There were no treatment-related mortalities or clinical signs throughout the course of the study in either sex. There were no treatment-related changes on body weight parameters (Fig. 7), food consumption, or ophthalmological examination in either sex.

No treatment-related changes were noted in either sex on hematology parameters (Table 2). In females, a marginal increase in platelet count (+13%, $p \leq 0.05$) was observed when compared with the control. Due to the low magnitude of this change, it was considered to reflect a normal inter-individual variation and not to be a treatment-related effect.

No treatment-related changes were noted in either sex on clinical chemistry parameters (Table 3). In females, a tendency towards lower aspartate aminotransferase and alanine aminotransferase activities were observed (-24%, not statistically significant and -27%, not statistically significant, respectively) when compared with the control. These changes were not considered to be treatment-related in view of the variation of the individual values.

No treatment-related changes were noted in either sex on terminal body weight and organ weights (Table 4). In males, the absolute and relative prostate weights were statistically significantly lower when compared with the controls (-27 to -28%, $p \leq 0.01$). However, except for one animal (5.5 mg), the individual values were within the range of the historical control data (min 5.6 mg, max 21.1 mg). Moreover, there were no changes in weights of the seminal vesicles, testis and epididymis. In addition, these changes were not associated with any relevant macroscopic or microscopic changes. Therefore, these changes in prostate weights were not considered to be treatment-related. In females, absolute and relative kidney weights were statistically significantly lower than the controls (-5 to -7%, $p \leq 0.05$). Since these changes were of low magnitude and were not associated with any relevant macroscopic or microscopic changes, they were not considered

Table 2
Repeated 28-day oral toxicity study: hematology.

Males	Unit	RBC	HGB	HCT	MCV	MCH	MCHC	RET	%RET	PLT	WBC	NEU	LYM	%NEU	%LYM
		10^{12}/l	g/dl	l/l	fl	pg	g/dl	10^{12}/l	%	10^{9}/l	10^{9}/l	10^{9}/l	10^{9}/l	%	%
CONTROL GROUP	(n)	10	10	10	10	10	10	10	10	10	10	10	10	10	10
	Mean	10.126	14.99	0.5165	51.0	14.79	29.03	0.314	3.10	1209.5	5.33	0.42	4.70	7.8	88.2
	Std Dev	0.2616	0.269	0.0130	0.62	0.223	0.386	0.0244	0.294	134.61	1.025	0.099	0.906	1.11	1.22
HPPD W336 GROUP	(n)	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	Mean	10.040	15.03	0.5174	51.6	14.99	29.07	0.321	3.20	1199.2	6.05	0.47	5.33	7.9	88.0
	Std Dev	0.4690	0.574	0.0151	1.14	0.333	0.412	0.0606	0.725	137.35	0.962	0.162	0.929	2.93	3.14
Females	Unit	RBC	HGB	HCT	MCV	MCH	MCHC	RET	%RET	PLT	WBC	NEU	LYM	%NEU	%LYM
		10^{12}/l	g/dl	l/l	fl	pg	g/dl	10^{12}/l	%	10^{9}/l	10^{9}/l	10^{9}/l	10^{9}/l	%	%
CONTROL GROUP	(n)	10	10	10	10	10	10	10	10	10	10	10	10	10	10
	Mean	9.953	15.17	0.5154	51.8	15.25	29.41	0.360	3.63	978.0	4.86	0.41	4.25	8.2	87.5
	Std Dev	0.3600	0.323	0.0126	1.20	0.37	0.443	0.0476	0.546	127.49	0.716	0.163	0.563	2.06	1.76
HPPD W336 GROUP	(n)	10	10	10	10	10	10	10	10	10	10	10	10	10	10
	Mean	9.858	15.01	0.5112	51.9	15.24	29.38	0.352	3.57	1106.4[a]	4.78	0.38	4.20	7.9	87.9
	Std Dev	0.36	0.5	0.022	2	0.1	0.8	0.0235	0.275	145	0.921	0.140	0.813	1.84	1.87

HCT: Hematocrit, HGB: Hemoglobin concentration, LYM: Lymphocytes, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, MCV: Mean corpuscular volume, NEU: Neutrophils, PLT: Platelet count, RBC: Red blood cells, RET: Reticulocytes, WBC: White blood cells. Std dev: Standard deviation.
[a] The group mean is significantly different from the control at $p \leq 0.05$.

Table 3
Repeated 28-day oral toxicity study: clinical chemistry.

Males	Unit	TBIL	ASAT	ALAT	AP	NA	K	GLUC	UREA	CREA	CHOL	TPRO	ALB
		(μmol/L)	(IU/L)	(IU/L)	(IU/L)	(mmol/L)	(mmol/L)	(mmol/L)	(mmol/L)	(μmol/L)	(mmol/L)	(g/L)	(g/L)
CONTROL GROUP	(n)	10	10	10	10	10	10	10	10	10	10	10	10
	Mean	1.07	80.7	31.5	118.5	161.7	4.40	8.810	13.352	35.7	2.543	58.1	36.8
	Std Dev	0.241	27.67	15.18	13.02	1.49	0.275	1.4607	1.6658	2.26	0.2204	1.91	1.40
HPPD W336 GROUP	(n)	10	10	10	10	10	10	10	10	10	10	10	10
	Mean	1.30	86.3	30.5	121.4	161.0	4.37	9.625	13.401	36.2	2.522	57.8	36.3
	Std Dev	0.392	15.28	6.62	14.24	1.25	0.427	1.8995	2.6091	3.16	0.3196	1.87	1.25
Females	Unit	TBIL	ASAT	ALAT	AP	NA	K	GLUC	UREA	CREA	CHOL	TPRO	ALB
		(μmol/L)	(IU/L)	(IU/L)	(IU/L)	(mmol/L)	(mmol/L)	(mmol/L)	(mmol/L)	(μmol/L)	(mmol/L)	(g/L)	(g/L)
CONTROL GROUP	(n)	10	10	10	10	10	10	10	10	10	10	10	10
	Mean	1.24	135.1	46.7	174.6	160.1	4.27	8.298	13.393	36.2	1.874	56.8	36.9
	Std Dev	0.497	50.34	18.11	14.95	1.91	0.313	1.0929	2.1051	2.49	0.2403	2.82	1.91
HPPD W336 GROUP	(n)	10	10	10	10	10	10	10	10	10	10	10	10
	Mean	1.29	103.0	34.2	163.6	159.7	4.29	9.174	13.576	36.2	1.888	57.2	37.0
	Std Dev	0.247	30.17	12.05	9.58	1.16	0.273	1.6702	2.4662	2.20	0.1336	2.44	1.76

ALAT: Alanine aminotransferase activity, ALB: Albumin concentration, AP: Alkaline phosphatase activity, ASAT: Aspartate aminotransferase activity, CHOL: Total cholesterol concentration, CREA: Creatinine concentration, GLUC: Glucose concentration, K: Potassium concentration, NA: Sodium concentration, TBIL: Total bilirubin concentration, TPRO: Total protein concentration, UREA: Urea concentration. Std dev: Standard deviation.

Table 4
Repeated 28-day oral toxicity study: terminal body weight (TBW), absolute organ weights.

Males	Unit	TB W	ADRENAL	BRAIN	EPIDIDYMIS	HEART	KIDNEY(S)	LIVER	PROSTATE	SPLEEN	TESTIS	THYMUS	SEMINAL VESICLE(S)
		(g)	(g)	(g)	(g)	(g)	(g)	(g)	(g)	(g)	(g)	(g)	(g)
CONTROL GROUP	(n)	10	10	10	10	10	10	10	10	10	10	10	10
	Mean	21.25	0.00403	0.4499	0.063	0.1313	0.2992	0.921	12.22	0.06428	0.1895	0.0291	235.12
	Std Dev	1.272	0.000585	0.01547	0.0039	0.01148	0.02932	0.1242	2.401	0.009512	0.00846	0.00468	23.612
HPPD W336 GROUP	(n)	10	10	10	10	10	10	10	10	10	10	10	10
	Mean	21.24	0.00401	0.4480	0.061	0.1264	0.2999	0.886	8.83[a]	0.06536	0.1813	0.0288	235.57
	Std Dev	1.380	0.000354	0.01162	0.0036	0.01584	0.03030	0.1040	2.783	0.013704	0.01363	0.00445	32.310

Females	Unit	TB W	ADRENAL	BRAIN	HEART	KIDNEY(S)	LIVER	OVARY(IES)	SPLEEN	THYMUS	UTERUS
		(g)	(g)	(g)	(g)	(g)	(g)	(g)	(g)	(g)	(g)
CONTROL GROUP	(n)	10	10	10	10	10	10	10	10	10	10
	Mean	17.44	0.00653	0.4518	0.1179	0.2519	0.844	0.0069	0.08096	0.0419	0.091
	Std Dev	0.703	0.000750	0.01151	0.00996	0.01424	0.1001	0.00135	0.010912	0.00607	0.0246
HPPD W336 GROUP	(n)	10	10	10	10	10	10	10	10	10	10
	Means	17.60	0.00656	0.4469	0.1111	0.2362[b]	0.774	0.0064	0.08476	0.0403	0.123
	Std Dev	0.631	0.000585	0.01333	0.00718	0.01508	0.0638	0.00133	0.009619	0.00685	0.0608

[a] The group mean is significantly different from the control at $p \leq 0.01$; Std dev: standard deviation.
[b] The group mean is significantly different from the control at $p \leq 0.05$; Std dev: standard deviation.

to be treatment-related. In addition, liver to body weight ratio was statistically significantly lower than the controls in females (-9%, $p \leq 0.05$). This change was observed in isolation and was considered not to be treatment-related.

No treatment-related macroscopic changes were observed in treated animals when compared with controls. No treatment-related microscopic changes were observed in treated animals when compared with controls. All the microscopic findings were those commonly observed in mice of this strain and age kept under laboratory conditions.

Therefore, HPPD W336 administered by gavage to C57BL/6J mice for at least 28 days, at the measured dose of 1000 mg/kg/day did not induce any treatment-related changes. In particular, no changes were observed on *e.g.* bilirubin concentration, reticulocyte count, hemoglobin concentration, lactate dehydrogenase activity, or bone marrow histology, which further supports the absence of hemolytic activity of HPPD W336 *in vivo*.

3.7. The modification of the Pseudomonas fluorescens HPPD at position 336 has negligible impact on its substrate specificity

HPPD W336 expressed in MST-FGØ72-2 soybean contains a single amino acid substitution compared with the sequence of the non-modified HPPD protein from *P. fluorescens*. To verify whether this amino acid substitution altered the substrate specificity, comparative studies were conducted between the *P. fluorescens* HPPD, HPPD W336 and native soybean HPPD expressed and purified from *E. coli*.

k_{cat} -values for non-modified HPPD and HPPD W336 were 4.0 ± 1.4 and 1.9 ± 0.4 s^{-1}, respectively and K_m-values for non-modified HPPD and HPPD W336 were 262 ± 54 and 262 ± 67 μM, respectively. This implies that the substitution has a slight impact on the conversion rate of 4-HPP into HGA, but not on the affinity of the *P. fluorescens* HPPD enzyme for 4-HPP.

A range of α-keto acids, such as PP (Lindstedt and Odelhög, 1987), KIC (Baldwin et al., 1995), KMTB (Crouch et al., 1997) and 3,4- dHPP (Fellman et al., 1972), have been described in the literature to be substrates for HPPD, in addition to their potential relevance for

Table 5
Relative reaction rates of enzymatic conversion of alternative substrates by different HPPD enzymes in relation to their enzymatic conversion of the natural substrate 4-HPP.

Substrate	Reaction product	Reference	Relative reaction rate (%)[a]		
			Wild type *P.fluorescens* HPPD	HPPD W336	Native soybean HPPD
4-HPP	HGA	Rundgren, 1983	100	100	100
PP	2-hydroxyphenylacetate	Lindstedt and Odelhög, 1987	< 0.3[b]	< 0.3[b]	3.3
KIC	β-hydroxyisovaleric acid	Baldwin et al., 1995	< 0.3[b]	< 0.3[b]	1.7
KMTB	3-methylsulfinylpropionic acid	Crouch et al., 1997	< 0.3[b]	< 0.3[b]	1.8
3,4-dHPP	2,4,5-trihydroxyphenylacetic acid	Fellman et al., 1972	2.4	4.8	3.6

[a] Expressed as % of 4-HPP conversion.
[b] 0.3% is the detection limit of the method applied, corresponding to an absence of turnover.

biological pathways operative in plant species. In plants, PP is suggested to act as an intermediate in alkaloid biosynthesis in *Solanaceae* (Humphrey and O'Hagan, 2001) and as a precursor of a floral scent component in *Rosa* (Watanabe et al., 2002). KIC has been suggested to be an intermediate in leucine catabolism in plants (Anderson et al., 1998), while KMTB has been suggested to be an intermediate in the methionine cycle in plants (Miyazaki and Yang, 1987). 3,4-dHPP has been described to be an intermediate in the synthesis of rosmarinic acid, a caffeic acid ester which accumulates in some plants, presumably as a defense compound (Petersen et al., 2009).

The reaction rates of the non-modified HPPD from *P. fluorescens*, HPPD W336 and the native soybean HPPD with the natural substrate 4-HPP and the potential alternative substrates PP, KIC, KMTB and 3,4-dHPP were determined by means of a colorimetric HPPD enzymatic assay. Results were expressed as relative conversions compared with 4-HPP (Table 5). For PP, KIC and KMTB no detectable conversion was observed for non-modified HPPD and HPPD W336, while for native soybean HPPD very low values were observed for these compounds which suggests these might be alternative substrates for native soybean HPPD. Only 3,4-dHPP can be considered as an alternative substrate for all three HPPD proteins, since conversion rates were observed above the limit of quantitation.

The absence of conversion of PP as an alternative substrate for HPPD is in contrast to what was reported by Lindstedt and Odelhög (1987) stating that PP is a substrate for *Pseudomonas* strain P.J 874 HPPD with a K_m value of 0.5 mM. This earlier, conflicting result can be explained either by a difference of the substrate spectrum of the two *Pseudomonas* HPPD enzymes or by the possibility that the result from their 1987 study may be caused by traces of 4-HPP in the PP preparation used.

Taken together, the results demonstrate that the modification of the *P. fluorescens* enzyme has negligible impact on the substrate specificity spectrum and that the substrate spectrum of HPPD W336 appears to be narrower than that of the native soybean HPPD protein.

3.8. Expression of the HPPD W336 protein in MST-FGØ72-2 soybean has no impact on the aromatic amino acid, tocochromanol and homogentisate content

Basic nutrients of soybean were analyzed from seed produced at eight sites in the USA. These included amino acids and subsequent metabolites from the tyrosine degradation pathway including tocochromanols. Table 6 summarizes data obtained from the analysis of the non-transgenic counterpart, test herbicides-untreated MST-FGØ72-2 soybean, test herbicides-treated MST-FGØ72-2 soybean and six commercial soybean varieties.

Statistical analysis of the data did not indicate overall entry differences across the eight locations for any of the aromatic amino acids analyzed (tyrosine, phenylalanine and tryptophan). Furthermore, all observed values were in the range of the included commercial varieties and the ILSI Crop composition database (version 5.0, 2014; Table 6). This demonstrates that there is no biologically meaningful effect from over-expression of HPPD W336 on the aromatic amino acids which are all closely related to the HPPD pathway. The compositional analysis of the other 15 proteinogenic amino acids also showed no overall entry differences (except for lysine in the comparison between non-GM counterpart vs MST-FGØ72-2 soybean treated with test herbicides) and all mean values (including those for lysine) were well within the range of the commercial varieties and the ILSI Crop composition database (Table 2 of Dreesen et al., submitted).

Levels of α-, γ- and δ-tocotrienols were below LOQ (< 1.0 mg/kg fresh weight) for almost all of the samples analyzed (Table 6), hence the entry mean values were not considered to be different. For γ-tocopherol and β-tocotrienol, however, statistically significant differences were found in the combined sites analysis between the non-GM counterpart and MST-FGØ72-2 soybean not treated with test herbicides and the non-GM counterpart and MST-FGØ72-2 soybean treated with test herbicides. However, all tocochromanol mean values were in the range of the commercial varieties and, where available, within the ranges obtained from the ILSI Crop composition database or the FAO/WHO Food Standards (2009). Therefore, the entry differences observed for γ-tocopherol and β-tocotrienol are considered to be negligible from a biological and nutritional standpoint.

Additionally, HGA levels were assessed in grain from both test herbicide-treated and -untreated MST-FGØ72-2 soybean, the non-GM counterpart 'Jack' and three commercial soybean varieties from six US field trial sites conducted. For all samples, HGA levels were found to be below the LOQ (2.00 ppm; Table 6), indicating that there appears to be no accumulation of the product of the HPPD reaction. Furthermore, over-expression of HPPD W336 in MST-FGØ72-2 soybean does not seem to have an influence on the composition of soybean grain, specifically on the levels of aromatic amino acids, HGA and tocochromanols.

The absence of any unintended changes in the soybean seed composition and the absence of allergenic potential of MST-FGØ72-2 soybean were further supported by a chicken feeding study (data not presented) and a study on known endogenous soybean food allergens (Rouquié et al., 2010), respectively.

4. Discussion

The characterization studies, described in section 3.1, adequately demonstrate that HPPD W336 produced in *E. coli* was a suitable surrogate for HPPD W336 expressed in MST-FGØ72-2 soybean.

Overall, the safety assessment of HPPD W336 showed no evidence of potential allergenicity. The source organism is not an allergen (OECD, 1997). HPPD proteins are ubiquitous in nature, and they are not known to be allergenic. Of major importance, HPPD W336 does not share relevant sequence identities, nor similarities, with proteins that are known to be allergenic, despite applying regulatory E-value thresholds which are far above relevant E-value thresholds for screening and detecting for allergenic cross-reactivity, which are within

Table 6
Aromatic amino acid, tocopherol, tocotrienol and HGA levels of MST-FGØ72-2 soybean seed in relation to herbicide treatment and conventional soybean varieties.

Component	Non-GM counterpart[a] Not treated with herbicides-Entry A	MST-FGØ72-2 soybean Not treated with test herbicides - Entry B	MST-FGØ72-2 soybean Treated with test herbicides[b] - Entry C	Range commercial Soybean varieties [c]	ILSI Version 5.0 (2014) – Codex Alimentarius (2005) (4)	Comparison *t*-test Entry A vs B[e]	Comparison *t*-test Entry A vs C[e]
	Mean ± St Dev	Mean ± St Dev	Mean ± St Dev	(min-max)	(min-max)	p-value	p-value
Tyrosine (mg/g dw)	13.4 ± 0.7	13.5 ± 0.8	13.4 ± 0.6	12.0–15.7	7.4–23.2	0.333	0.941
Phenylalanine (mg/g dw)	19.9 ± 1.3	20.1 ± 1.5	20.0 ± 1.2	17.6–24.1	15.0–25.8	0.209	0.631
Tryptophan (mg/g dw)	5.90 ± 0.38	5.88 ± 0.31	5.84 ± 0.29	4.90–7.46	2.54–7.31	0.677	0.337
α-Tocopherol (mg/kg dw)	50.4 ± 13.0	53.3 ± 10.8	53.8 ± 7.8	29.6–83.4	1.93–127.38	0.271	0.200
β-Tocopherol (mg/kg dw)	5.91 ± 1.16	5.78 ± 1.20	5.86 ± 1.05	3.13–11.47	2–9.79	0.865	0.837
γ-Tocopherol (mg/kg dw)	237 ± 37	262 ± 29	268 ± 22	160–323	14–362	0.003[f]	< 0.001[f]
δ-Tocopherol (mg/kg dw)	67.9 ± 11.2	69.4 ± 14.7	69.3 ± 11.9	26.6–96.3	8.56–149	0.558	0.570
α-Tocotrienol (mg/kg dw)	< 1.0	< 1.0	< 1.0	< 1.0	0–13.8 [d]	N.A.[g]	N.A[g]
β-Tocotrienol (mg/kg dw)	2.96 ± 0.66	3.41 ± 0.54	3.40 ± 0.48	2.33–4.42	N.A.	0.002[f]	0.003[f]
γ-Tocotrienol (mg/kg dw)	< 1.0	< 1.0	< 1.0	< 1.0	0–20.6 [d]	N.A.[g]	N.A.[g]
δ-Tocotrienol (mg/kg dw)	< 1.0	< 1.0	< 1.0	< 1.0	0 [d]	N.A.[g]	N.A.[g]
HGA (ppm)	< 2.00	< 2.00	< 2.00	< 2.00	N.A.	N.A.[g]	N.A.[g]

St Dev = Standard Deviation.

[a] Non-GM counterpart is non-GM soybean line MST39 except for HGA. For the latter it is soybean variety Jack.

[b] foliar application of isoxaflutole (70 g ai/hectare) + glyphosate (Roundup PowerMAX; 1032 g ai/hectare) at 4–5 leaf stage.

[c] Either Stine® 35E23, Stine® 29E22 and Stine® 35E32 or Stine® 33E22, Stine® 31E22 and Stine® 30E32, except for HGA: Stine® 2500-2, Stine® 3300-2 and Stine® 3308-2.

[d] ILSI does not provide references for tocotrienol and HGA levels since these compounds are not considered as common analytes. Instead, converted reference ranges for α-, γ-, and δ-Tocotrienol were listed from the Codex Alimentarius (2005), assuming a seed fat content of 20%.

[e] *t*-test p-value: pairwise comparison to the non-GM conventional counterpart (entry A).

[f] a p-value < 0.05 was observed which indicates a significant difference between means.

[g] Parameters with more than one third of values < LOQ were excluded from the statistical analysis.

the range of 1×10^{-5} -1×10^{-6} (McClain, 2017). Indeed, cross-reactivity among known allergens is rare at < 50% identity and typically requires > 70% identity (Aalberse, 2000; Thomas et al., 2008). Furthermore, HPPD W336 was shown to be rapidly degraded in the SGF test, *i.e.* over 90% degraded in less than 30 s. While there is no absolute correlation between pepsin resistance and allergenicity, complete digestion of a protein greatly reduces exposure and the possibility of elicitation. Although it does not represent all physiological conditions of the stomach (*e.g.* infants, elderly people, patients under antacid medication), the SGF test is internationally recognized as a valuable tool in the allergenicity assessment of newly expressed proteins (EFSA, 2011, Delaney, 2015). Furthermore, HPPD W336 was rapidly digested in simulated intestinal fluid. The conclusion is that HPPD W336 has very low allergenic potential.

In addition to the lack of potential allergenicity, HPPD W336 showed no evidence of potential toxicity. *P. fluorescens* has a history of safe use in agricultural, pharmaceutical, and bioremediation applications (OECD, 1997; US-EPA, 2009). It is used as a growth-promoting agent and as a biopesticide on certain crops to prevent the growth of frost-forming bacteria on leaves and blossoms (Compant et al., 2005; Flieβbach et al., 2009; OECD, 1997; Raaijmakers et al., 2006; US-EPA, 2009; Wilson and Lindow, 1993), or diseases caused by fungi and nematodes (Haas and Défago, 2005; Hamid et al., 2003; Thrane et al., 2001). The US EPA has recognized these *P. fluorescence* strains as unlikely to cause any adverse health effects in humans (US-EPA, 2009). For pharmaceutical use, *P. fluorescens* produces the antibiotic mupirocin, which is used to prevent *Staphylococcus aureus* infections (Hothersall et al., 2007; Tacconelli et al., 2003). Finally, strains of *P. fluorescens* are used in bioremediation applications for their ability to degrade a wide variety of compounds, *e.g.*, naphthalene, chlorinated aliphatic hydrocarbons or styrene (OECD, 1997). As the HPPD proteins are ubiquitous in nature, HPPD is present in organisms commonly consumed as human food and none of these HPPD proteins are known to be toxic. The rapid degradation in simulated gastric and intestinal environments suggests that the potential exposure to HPPD W336 when ingested is likely to be very short. Similarly, HPPD W336 was rapidly inactivated by heat treatment. A loss of protein function generally correlates with a loss of native structure, suggesting that the processing of soybean into food or feed would alter the structure of the protein and therefore, lower the probability that the protein exerts toxic activity (if any) when consumed in food or feed. As expected, the HPPD W336 sequence identity search against the general protein database showed identities with HPPD proteins from various origins. Among them, a few HPPD proteins were annotated as hemolysins. However, it was shown that the hemolytic activity was due to the formation of hemolytic melanin-like pigment resulting from the oxidation of HGT (Wintermeyer et al., 1994; Chang et al., 1997; Hegedus and Nayak, 1994; Steinert et al., 2001). Therefore, the hemolytic activities only result indirectly from the HPPD activities of these proteins, and any HPPD protein would lead to the same results under these experimental conditions. To confirm this, an *in vitro* hemolysis test was performed in human blood. HPPD W336 did not cause hemolysis, as opposed to the alpha-hemolysin from *Staphylococcus aureus*. The absence of hemolytic activity of HPPD W336 was further confirmed in the acute toxicity study and in the repeated 28-day toxicity study, where no findings were observed on *e.g.* bilirubin concentration, reticulocyte count, hemoglobin concentration, lactate dehydrogenase activity, or bone marrow histology. Toxic proteins generally act through acute mechanisms, and therefore, an acute toxicity study is generally sufficient to assess the potential toxicity of proteins (Delaney et al., 2008). In the case of HPPD W336, the repeat dose 28-day toxicity study had an added value, since the repeated exposure and the expanded set of endpoints allowed assessing the potential hemolytic activity of the protein further.

To investigate whether the introduction of HPPD W336 in soybean would induce any metabolic shift, the substrate specificity of HPPD W336 was compared with that of the non-modified HPPD from *P. fluorescens* and the endogenous soybean HPPD. Determined K_m and K_{cat}-values for the non-modified HPPD and HPPD W336 using 4-HPP as a substrate clearly demonstrated that the amino acid substitution has an impact on the conversion rate of 4-HPP to HG, but not on the affinity of the enzyme for 4-HPP. This is in line with the findings of Matringe et al. (2005), who determined Michaelis constants in relation to the interaction of HPPD W336 and non-modified HPPD with the inhibitor DKN, which binds to the same site in HPPD as 4-HPP. Here, the substitution was shown to have a moderate impact on K_m and V_{max}, but with a strong negative effect on DKN sensitivity as revealed by a K_{on} value of $4.4 \times 102\,M^{-1}s^{-1}$. Taken together, both studies suggest that the side chain of the introduced tryptophan in HPPD W336 hampers accessibility of the HPPD active site for the substrate and the inhibitor.

Some of the α-keto acids tested were confirmed as alternative substrates for the HPPD enzymes. However, these results only demonstrate that quantifiable reaction rates were obtained with the alternative substrates under *in vitro* conditions, but at a very slow reaction rate. The assay conditions included a substrate concentration (0.5 mM) which is at saturation for HPP, high enzyme concentrations (7.5–10-fold the native soybean HPPD enzyme concentration used with the natural substrate 4-HPP), and extended incubation times, without competing substrates. Such conditions, predicted to favor a reaction, are not expected to occur *in planta*. Therefore, the observed relative reaction rates are highly unlikely to be relevant *in planta*, even if the alternative substrates were to be present. Nevertheless, it can be concluded that the substrate spectra of non-modified *P. fluorescens* HPPD and HPPD W336 are more narrow than native soybean HPPD. Consequently, the introduction of HPPD W336 in MST-FGØ72-2 soybean plants is not expected to cause a shift in the substrate specificity of the native soybean HPPD enzyme. Hence, it is considered highly unlikely that expression of HPPD W336 leads to any unintended metabolic effects in MST-FGØ72-2 soybean.

Given the critical roles of HPPD in plant metabolism, over-expression of HPPD W336 in MST-FGØ72-2 soybean may be expected to alter levels of aromatic amino acids, particularly tyrosine, HGA, and subsequent metabolites such as the tocochromanols. Compositional analyses were conducted on field produced grain from MST-FGØ72-2 soybean (both untreated and treated with test herbicides), a non-GM counterpart, and six commercial soybean varieties. All results were compared with the ILSI Crop composition database (2014) and the FAO/WHO food standards (2009). No differences in tyrosine levels were observed for test herbicides-treated or -untreated MST-FGØ72-2 soybean compared with the non-GM counterpart. This is as expected since tyrosine biosynthesis is tightly regulated in plants by feedback mechanisms, mainly to ensure the correct carbon flux is directed towards phenylalanine (Maeda and Dudareva, 2012). Furthermore, elevated levels of tyrosine were proposed as one of the reasons for phytotoxicity of HPPD-inhibiting herbicides (Prisbylla et al., 1993; Pallett et al., 1998). Based on this observation and had there been any increase in tyrosine levels in MST-FGØ72-2 soybean, phytotoxicity would have been observed in its phenotype. The non-elevated tyrosine levels in addition to the lack of phytotoxicity, indicate that there appears to be no influence of HPPD W336 over-expression on tyrosine accumulation. Similarly, there were no differences in phenylalanine and tryptophan levels between MST-FGØ72-2 soybean and the non-GM counterpart indicating that the expression of HPPD also had no impact on any of the aromatic amino acids.

The ranges in the compositional studies are in line with the natural variation observed for soybean varieties and supported by the ILSI Crop compositional database (2014) and the FAO/WHO food standards (2009). The fact that β-tocopherol and tocotrienols levels are very low in MST-FGØ72-2 soybean is also described for over-expression of modified oat HPPD protein in other soybean lines (Kramer et al., 2014). There were statistically significant differences for γ-tocopherol and β-tocotrienol, which were demonstrated to be negligible from a biological perspective and the values were within the ranges from the six

commercial reference varieties included in the study and from the external reference sources. Consequently, the specific differences in this study have no biological or nutritional relevance. These findings are largely supported by an extensive literature review performed by Mène-Saffrané and DellaPenna (2010). Expression of the HPPD enzyme alone in a number of crops, including rapeseed, rice, tobacco, *Arabidopsis* and soybean does not or only modestly increase tocopherol levels (Falk et al., 2003; Farré et al., 2012; Matringe et al., 2005; Tsegaye et al., 2002; Raclaru et al., 2006; Kramer et al., 2014). These reports provide further evidence of a tight regulation of the biosynthesis and subsequent metabolism of tyrosine and HGA in plant cells. Co-expression of prephenate dehydrogenase (PDH) and HPPD appears necessary to elevate tocochromanols in plants. Expression of yeast PDH and *Arabidopsis*HPPD in tobacco plants markedly enhanced the accumulation of tocotrienols (Matringe et al. 2005; Rippert et al. 2004). Similar findings have been reported for GM soybean and *Arabidopsis* plants co-expressing bacterial PDH and *Arabidopsis* HPPD (Karunanandaa et al., 2005). Further studies have shown that expression of the enzymes that convert HGA into tocotrienols and tocopherols is necessary to impact their levels in plant cells. Elevated levels of these vitamin E homologues were observed when homogentisate prenyl transferase (HPT, Fig. 1) was co-expressed with γ-tocopherol methyl transferase in *Arabidopsis* (Collakova and DellaPenna, 2003), when HPPD was co-expressed with both HPT and tocopherol cyclase in *Brassica napus* (Raclaru et al., 2006) or when homogentisate geranyl geranyl transferase (HGGT; Fig. 1) was over-expressed alone in tobacco (Tanaka et al., 2015). The findings from the composition analyses as well as those from the literature show that over-expression of HPPD alone does not have any significant impact on tocochromanol levels, due to tight metabolic control of tyrosine levels in plant cells as well as regulation of HGA conversion into prenylquinones.

In addition to the anabolic role of the tyrosine degradation pathway, a catabolic part of the pathway exists where HGA is converted into fumarate and acetoacetate. In this study, fumarate or acetoacetate levels were not determined. However, it is not expected that their levels would be influenced by the overexpression of HPPD W336 since tyrosine, HGA and tocopherol levels were not changed in MST-FGØ72-2 soybean. Furthermore, both fumarate and acetoacetate are considered as intermediates which are recycled into the plant's primary energy metabolism (Bolton, 2009). All 18 amino acids are recycled through these compounds and the other five intermediates acetyl-CoA, α-ketoglutarate, succinyl-CoA, pyruvate and oxaloacetate. The fact that the amino acid levels were unchanged in MST-FGØ72-2 soybean supports the assumption that the levels of fumarate and acetoacetate should remain unchanged as well. This assumption is supported by Clarke et al. (2013) who did not find any significant differences in fumarate levels in soybean lines overexpressing a modified oat HPPD.

Taken together, compositional analysis data do not indicate that expression of HPPD W336 in MST-FGØ72-2 soybean leads to any unintended changes, in particular in the anabolic and catabolic pathways involving the HPPD enzyme. MST-FGØ72-2 soybean is as safe and nutritious as its non-GM counterpart. This conclusion is supported by results from feeding studies on poultry and rodents which demonstrate that MST-FGØ72-2 soybean is safe for food and feed consumption (data not shown).

In conclusion, the work described here demonstrates the safety of HPPD W336 and MST-FGØ72-2 soybean. This opinion is supported by favorable food, feed and environmental risk assessments of the HPPD W336 protein and MST-FGØ72-2 soybean in many countries (CERA, 2016).

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