9-5-96

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

SEP - 5 1996

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Review of Data on Product Identity, Acute Oral Toxicity and *In vitro* Digestibility of Glyphosate Oxidoreductase (GOX) Submitted by Monsanto Co.

TO: Michael Mendelsohn
Regulatory Action Leader
Biopesticides & Pollution Prevention Division
(7501W)

FROM: John L. Kough, Ph.D., Biologist
Biopesticides & Pollution Prevention Division
(7501W)

THROUGH: Roy D. Sjoblad, Ph.D., Senior Scientist
Biopesticides & Pollution Prevention Division
(7501W)

DATA REVIEW RECORD

Inert Ingredient:	Glyphosate oxidoreductase (GOX) from *Achromobacter* sp.
Product Name:	Round-up Ready™ herbicide resistant plants
Company Name:	Monsanto Corporation, St.Louis, MO
ID No:	6E04657
Submission No:	S504252
Chemical No:	006401
DP Barcode:	D225527
MRID:	439037-01 through 08

THIS DOCUMENT CONTAINS FIFRA CONFIDENTIAL BUSINESS INFORMATION

ACTION REQUESTED

To review the information submitted on the GOX enzyme to justify a food use inert clearance.

BACKGROUND

The information provided concerns an enzyme employed by Monsanto to provide resistance to their glyphosate herbicide. This herbicide resistance trait is used both as a single agronomic trait to allow use of glyphosate on growing crops and as a marker gene in plant cell cultures to provide a selection tool for detecting successful introduction of a desired trait such as insect resistance or increased sugar content.

BPPD RECOMMENDATIONS

The information is sufficient to support an exemption from the requirement of a food tolerance for the GOX protein. The information provided on acute oral toxicity and rapid degradation in digestive fluids would support the inclusion of this protein on the inerts list cleared for use in all plant species. It should be noted that this recommendation is based on the current understanding of protein toxicity and that these studies are being reviewed in the absence of established EPA testing guidelines for proteins. Neither the environmental fate of this gene nor the effect of its gene product on non-target organisms has been addressed in this submission.

SUMMARY OF REVIEWS

439037-01: GOX catalyzes the conversion of glyphosate to aminomethylphosphonic acid (AMPA) and glyoxylate in a 1:1 stoichiometry while consuming ½ mole of oxygen as a cosubstrate. GOX requires flavin adenine dinucleotide (FAD) and magnesium for activity; therefore, it is more appropriately designated an apoenzyme. ^{14}C glyphosate conversion to $^{14}AMPA$ with the release of glyoxylate only in the presence of GOX confirms that GOX activity is responsible for the removal of the terminal two carbons at the amide bond. IDA and 2-methyl glyphosate are the only other substrates for GOX of the L and D-amino acids and related compounds tested.

CLASSIFICATION: Acceptable.

439037-02: The isolation, cloning and sequencing of the GOX glyphosate to AMPA activity from a glyphosate-degrading bacterial culture collection was described. The GOX DNA sequence was altered in GC content to yield higher plant expression. These changes also altered three amino acids in the sequence and added a four-amino

acid sequence to the N-terminus, but did not negatively affect the enzymatic activity. The sequence was compared to other proteins, and the highest similarity was to D-amino acid dehydrogenases, especially in the FAD binding region.
CLASSIFICATION: Acceptable.

439037-03: The large scale production of an *E. coli* strain expressing the GOX enzyme is described.
CLASSIFICATION: None assigned. This information is not necessary for risk assessment.

439037-04: The two GOX variants, GOX(M4-C1) and GOXv247(M4-C1), expressed in *E. coli* showed similarity to the native GOX protein. These similarities are seen as comparable molecular weights, immunoreactivity, amino acid sequence and enzymatic activity.
CLASSIFICATION: Acceptable.

439037-05: The *E.coli* expressed GOX protein was shown to have a comparative activity against IDA and glyphosate similar to that expressed in corn line 423-06-1. Corn lines MON 802, 805, 830, 831, 832, 813 and 814 all had detectable levels of immunoreactive bands that co-migrated with the GOX protein in the control extracts. These data support the use of the microbially produced GOX as a surrogate in the acute oral toxicity and digestibility tests.
CLASSIFICATION: Acceptable.

439037-06: Both variants of the GOX protein (GOX(M4-C1) & GOXv247(M4-C1)) are rapidly degraded in simulated gastric fluid (GF) and simulated intestinal fluid (IF). After a fifteen-second incubation in GF, both variants have less than 90% of their initial protein epitopes by western blot analysis. Enzyme activity loss is also greater than 90% in both GOX variants when assayed after a one minute incubation in GF. Similar results are seen in simulated intestinal fluid (IF). Western blot assays show that both variants are greater than 90% degraded by 30-second incubation in IF. However, the enzyme activity assays show that the GOX(M4-C1) activity lasts longer in IF than variant GOXv247. After a 10-minute IF incubation, the activity decreased to about 48% of initial for GOX(M4-C1) whereas GOXv247 was already greater than 90% inactive.
CLASSIFICATION: Acceptable.

439037-07: The highest dose administered approached 100mg/kg bodyweight target for the high dose. The actual values were 91.3mg/kg for GOX(M4-C1) and 104mg/kg for GOXv247(M4-C1).
CLASSIFICATION: Acceptable.

439037-08: No animals died during the study. The weights of the mice at eight days after dosing were not significantly different between treatment groups. Clinical signs were not observed in any group during the nine day observation period prior to sacrifice. None of the findings at gross necropsy indicate an abnormal, treatment-related pathology.
CLASSIFICATION: Acceptable.

439037-09: None of the amino acid sequences of known allergens or proteins involved in coeliac disease were shown to have similarity to the GOX protein as defined by eight identical and contiguous amino acids in a sequence.
CLASSIFICATION: Acceptable.

BPPD COMMENTS
In totality, these studies show no acute oral toxicity or predictable allergenicity hazard for this protein expressed in plants. Two findings that are remarkable are found in the *in vitro* digestibility studies are: 1) GOXv247 displays a more rapid degradation in intestinal fluid compared to unaltered GOX, apparently due to a single amino acid substitution; and 2) antibody recognition is lost prior to a significant loss of enzyme activity indicating that western blots may not always accurately track functional protein degradation.

One feature of the submission that is questionable is the assertion of lack of allergenicity can be established by comparison of sequences to known allergens. While this is the best approximation at present, there is no scientific basis to assume that the presence of eight contiguous and homologous amino acids in a protein will predict its allergenicity. The assumption is based on the finding that the presence of an eight amino acid sequence in one allergen was associated with the epitope responsible for IgE recognition. Alteration of this sequence reduced IgE binding and hence allergenicity. The converse experiment, to introduce the sequence into a non-allergenic protein and create an allergen, has not been attempted experimentally.

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD JLK

Secondary Reviewer: Sheryl K. Reilly, Ph.D., Biologist, BPPD SKR

STUDY TYPE:	Product Characterization
MRID NO:	439037-01
CHEMICAL NO:	006401
TEST MATERIAL:	GOX enzyme
STUDY NO:	MSL-13234
SPONSOR:	Monsanto Co., St.Louis, MO
TESTING FACILITY:	Monsanto Co., St. Louis, MO
TITLE OF REPORT:	Characterization of Glyphosate Oxidoreductase
AUTHORS:	Stephen R. Padgette, Mary L. Taylor, Gerard F. Barry Thomas Huber, Leslie A. Harrison & Ganesh M. Kishore
STUDY COMPLETED:	May 26, 1994
CONCLUSION:	GOX catalyzes the conversion of glyphosate to aminomethylphosphonic acid (AMPA) and glyoxylate in a 1:1 stoichiometry while consuming ½ mole of oxygen as a cosubstrate. GOX requires flavin adenine dinucleotide (FAD) and magnesium for activity; therefore, it is more appropriately designated an apoenzyme. ^{14}C glyphosate conversion to $^{14}AMPA$ with the release of glyoxylate only in the presence of GOX confirms that GOX activity is responsible for the removal of the terminal two carbons at the amide bond. IDA and 2-methyl glyphosate are the only other substrates for GOX of the amino acids and related compounds tested.
CLASSIFICATION:	Acceptable.

STUDY DESIGN

To describe the biochemical features of GOX catalyzed reactions and the purification process to obtain the toxicity test material.

TEST MATERIAL

Bacterial GOX fermentation: *Escherichia coli* strain JM101 or GB993 containing either pMON17010 or pMON17061, respectively, were grown in 100L fermenters and induced to produce GOX. The cells were collected by centrifugation and washed with 0.9% NaCl. Refractile bodies were then extracted from the cells by resuspension in

cracking buffer (50mM KH_2PO_4, 1mM DTT, 10% glycerol, 1mM PMSF, 1mM BAM & 5mM ACA, pH 7.2) passage through a french press and centrifugation. Several differential centrifugations were done to obtain the final solution enriched in GOX as a 20-40% ammonium sulfate cut. This 20-40% ammonium sulfate suspension was stored at -80°C until processed further as described below.

TEST METHODS

Enzyme Assays: The activity of the GOX enzyme was characterized by monitoring the accumulation of glyoxylate, one of the GOX reaction products. The glyoxylate product is derivitized with 2,4-dinitrophenylhydrazine (DNPH), which yields a product that can be monitored by spectrophotometric absorbance at 520 nm when adjusted to a basic pH. A standard curve was generated for glyoxylate ranging from 0.2mM to 1.0mM for determining the glyoxylate concentration in the reaction mixture. Enzyme activity was also monitored by oxygen uptake, once the oxygen dependency of the GOX reaction was verified. The oxygen depletion of the test solution was followed by a Gilson 510 oxygraph calibrated for 222μM O_2 full scale deflection. Finally, an assay was used to confirm the proposed GOX activity against glyphosate by utilizing 3-^{14}C glyphosate and monitoring its conversion to radiolabelled aminomethyl-phosphonic acid (AMPA), which along with glyoxylate are the products of the GOX reaction with glyphosate. The reaction was monitored by appearance of radioactivity in the AMPA fraction, which under HPLC (Synchropak AX100 with 0.065M isocratic potassium phosphate, pH 5.5) elutes at 6 minutes versus 11 minutes for glyphosate.

Preparation of GOX-specific antibody: The refractile bodies containing GOX obtained from the crude bacterial extracts were dissolved in Laemmli cracking buffer and separated into bands by SDS-PAGE. The bands close to the predicted 45 kD molecular weight were cut out and mixed 1:1 with Freund's complete adjuvant and 1 to 5 ml used to immunize a goat by intramuscular injection. Boosts (days 20, 46 and 68) were done in the same manner with Freund's incomplete adjuvant. Blood samples were taken at regular intervals to determine the specific titer and the animal was bled on day 74. The IgG fraction was separated by use of a Pharmacia Protein G column according to manufacturer's instructions.

Purification of GOX: GOX purity was followed by monitoring GOX activity using the glyoxylate DNPH spectrophotometric assay and the

protein concentration. The first purification step (after cell lysis and ammonium sulfate precipitation) was testing a series of purification schemes: hydroxylapatite/MonoQ, S-1000/hydroxylapatite and S-1000/alkyl Superose, and finally a density gradient centrifugation method. All methods used the 20-40% ammonium sulfate cut frozen at -80°C as the starting material.

Hydroxylapatite/MonoQ: A 50 gm sample of the 20-40% cut containing from 2.09 to 2.18 gm protein was dissolved in MQ buffer A (10mM TRIS HCl, 10% glycerol &1 mM DTT, pH 7.4) and dialyzed 3 times versus that buffer to remove the salt. A sample of this solution containing 1 gm protein was loaded onto a 200ml Bio-Rad hydroxylapatite column equilibrated with HA buffer A (10mM KH_2PO_4, 1mM DTT & 10% glycerol, pH 7.4). The column was then washed with HA buffer B (0.25mM KH_2PO_4, 1mM DTT & 10% glycerol, pH 7.4), eluted with HA buffer C (1.0mM KH_2PO_4, 1mM DTT & 10% glycerol, pH 7.4). Fractions of 8.2ml were collected, tested for GOX activity and fractions 17 through 26 were pooled. This solution was dialyzed three times against MQ buffer A + 10μM FAD then three times against MQ buffer A alone and loaded onto a Pharmacia MonoQ 10/10 column. The protein was eluted by a MQ buffer A/MQ buffer B (10mM TRIS Hcl, 10% glycerol, 1 mM DTT & 1M KH_2PO_4, pH 7.4) gradient (0-100% MQ buffer B in 80 minutes) and collected in 5 ml fractions. Three pools were made based on GOX specific activity: pool 1= fractions 9-11 & 32-37; pool 2= fractions 12-15 & 26-31 and pool 3= fractions 16-25. Pool 3 (fractions 16-25) was concentrated from 30 ml to 1 ml by a Amicon YM10 membrane, glycerol was added to 20% and the preparation was frozen at -20°C until used for further analysis.

S-1000/hydroxylapatite and S-1000/alkyl superose: Using an *E. coli* GB993:pMON17061 cell paste, GOX-active fractions collected from the hydroxylapatite column described above were dialyzed (10kD molecular weight cutoff) twice against GOX storage buffer (100mM TRIS, 50% glycerol, pH 7.4), then stored at -20°C. When needed, the solution was diluted in S-1000 buffer (100mM TRIS Cl, 100mM NaCl, pH 7.4) and run on a 2.6 x 88 cm Sephacryl S-1000 column. Fractions were collected and analyzed for GOX activity and SDS-PAGE. A 78.8mg portion of the original 20-40% cut (9ml) was also loaded directly onto a S-1000 column after dilution to 10 ml with S-1000 buffer. GOX was eluted with S-1000 buffer, GOX positive fractions were pooled and concentrated to 8 ml or 7.8mg protein. This solution was dialyzed into AS buffer A (50mM TRIS-Cl, 1.2M ammonium sulfate, 10% glycerol, 1mM DTT, pH 7.4), loaded onto an

Alkyl Superose 5/5 column and eluted with a gradient of 100% AS buffer A to 100% AS buffer B (50mM TRIS-Cl, 10% glycerol, 1mM DTT, pH 7.4) in 45 minutes. Fractions were analyzed by GOX activity and SDS-PAGE. After the gradient was completed additional GOX was eluted with 50% ethylene glycol in AS buffer B.

Density Gradient: Using cell paste from *E. coli* GB993:pMON17061, the 20-40% cut was dialyzed twice against buffer A (100mM TRIS-Cl, 1mM DTT, 1mM EDTA, 10μM FAD, 1mM benzamidine & 20% glycerol) with a 30kD cutoff, then divided into 10 ml aliquots. An aliquot (approximately 134-175 mg protein) was thawed, diluted in buffer A minus glycerol to 200 ml, and centrifuged and washed in a Filtron 300K to yield 30 ml of supernatant. One ml of this supernatant was loaded onto the top of a six-step sucrose gradient (5ml of 60%, 5ml of 50%, 5ml of 45%, 5ml of 40% and 13.5ml of 30% sucrose solution in a 40 ml Ultra-Clear™ centrifuge tube). The tubes were centrifuged at 100,000 xg for 20 hr and 2ml fractions were obtained by puncturing the bottom of the tube with a needle and collecting drops. Similar fractions were pooled and the pools were sampled for GOX activity and protein concentration.

RESULTS

These studies show that GOX catalyzes the conversion of glyphosate to aminomethylphosphonic acid (AMPA) and glyoxylate in a 1:1 stoichiometry while consuming ½ mole of oxygen as a cosubstrate. GOX requires flavin adenine dinucleotide (FAD) and magnesium for activity; therefore, it is more appropriately designated an apoenzyme. ^{14}C glyphosate conversion to $^{14}AMPA$ with the release of glyoxylate only in the presence of GOX confirms that GOX activity is responsible for the removal of the terminal two carbons at the amide bond.

Biochemical studies indicate that both magnesium and manganese ions increase GOX activity, whereas cuprous ion decreased activity 50%. Ferric ion had a stimulatory effect, whereas ferrous ion had no effect on the GOX reaction in the presence of magnesium. The appK_m for glyphosate of the GOX enzyme is 18-25mM. The GOX reaction can proceed in the absence of oxygen, if ubiquinone is present to function as an electron acceptor. The temperature and pH optima for GOX are 50°C (the activity decrease above this temperature is due to inactivation of the enzyme itself) and pH 6.5 (the enzyme is inactivated by pH above 8.8). GOX's highest catalytic activity is the oxidation of iminodiacetic acid (IDA) with an appK_m of 3 mM IDA,

followed by glyphosate then 2-methyl glyphosate. GOX was found to have no detectable activity against the following substrates: N-methyl-glyphosate, glycine oxalamide, sacrosine, glycine, and the L & D forms of alanine, phenylalanine, aspartic acid, glutamic acid and methionine.

Gel filtration demonstrated that GOX is often found as an aggregate with an apparent molecular weight of over 300 kD. These aggregates are formed both in the refractile bodies in *E. coli* and during the purification process. The actual molecular weight of GOX is approximately 41.6 kD and this species is found in western blots of purified extracts. GOX activity is not affected by a wide range of detergents (deoxycholic acid, sodium sarkosyl, cetylpyridinium chloride, CHAPS, N-dodecylglycopyranoside, Nonidet & Triton X-100). However, SDS, Tween 20 and dodecyltrimethyl ammonium bromide all reduced activity.

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD JLK

Secondary Reviewer: Sheryl K. Reilly, Ph.D., Biologist, BPPD SKR

STUDY TYPE:	Product Characterization
MRID NO:	439037-02
CHEMICAL NO:	006401
TEST MATERIAL:	GOX gene & expressed protein
STUDY NO:	MSL-13245
SPONSOR:	Monsanto Co., St.Louis, MO
TESTING FACILITY:	Monsanto Co., St. Louis, MO
TITLE OF REPORT:	Cloning and Expression in *Escherichia coli* of the glyphosate-to-aminomethylphosphonic acid degrading activity from *Achromobacter* sp. strain LBAA
AUTHORS:	Gerard F. Barry, Mary L. Taylor, Stephen R. Padgette, Kathryn H. Kolacz, Laurence E. Hallas, Guy della-Cioppa & Ganesh M. Kishore
STUDY COMPLETED:	May 9, 1994
CONCLUSION:	The isolation, cloning and sequencing of the GOX glyphosate to AMPA activity from a glyphosate degrading bacterial culture collection was described. The GOX DNA sequence was altered in GC content to yield higher plant expression. These changes also altered three amino acids in the sequence and added a four amino acid sequence to the N-terminus but did not negatively affect the enzymatic activity. The sequence was compared to other proteins and the highest similarity was to D-amino acid dehydrogenases especially in the FAD binding region.
CLASSIFICATION:	Acceptable.

STUDY DESIGN

The isolation, cloning and expression of a glyphosate detoxifying enzyme from a collection of glyphosate degrading bacteria into *E. coli* is described.

TEST MATERIALS & METHODS

The glyphosate degrading bacterium with the highest activity in the collection was an *Achromobacter* species strain LBAA. The chromosomal DNA from this bacterium was subject to a partial

*Hind*III digestion, ligated into a plasmid, packaged into lambda phage and used to infect an *E. coli* strain for screening the library. Once glyphosate-utilizing colonies were identified, glyphosate degradation activity was assayed in cell free extracts using the glyoxylate-DNPH assay. Once glyphosate degradation was detected in a clone, the 6.4kb insert was further digested with endonucleases that cut infrequently in the insert and finally a 1.8kb fragment was found to be the smallest to contain the active moiety.

To identify the proteins encoding the glyphosate degrading activity, a T7 expression system was used for the 1.8kb insert. An ^{35}S-labelled protein of approximately 45kD was detected on an SDS-PAGE gel exposed to X-ray film. This insert was then sequenced in both directions and the sequence scanned for open reading frames. The sequence was subsequently altered in G+C content to improve expression in plants by using plant-preferred codons, reducing likely polyadenylation sites and possible hairpin loops in the expressed mRNA. These changes in the DNA yielded three amino acid substitutions and an additional four amino acids at the N-terminus resulting from signal protease activity in the chloroplast. The changes are at amino acid residue 84 (a serine to glycine), at residue 153 (a lysine to arginine) and at residue 334 (a histidine to arginine). The additional four amino acids at the N-terminus are: cysteine, methionine, glutamine and alanine, which are left over from the action of the signal protease found in the chloroplast. (phone conversation with P. Sanders, September 3, 1996)

The amino acid sequence of GOX was screened for homology with other known proteins using an undescribed homology algorithm. The closest match was with D-amino acid dehydrogenase, with lesser relatedness to sacrosine oxidase, D-amino acid oxidase and monoamine oxidase. The region of greatest homology was in the FAD-binding region of these enzymes. Given the characterization data demonstrating a requirement for this co-factor in GOX activity, the FAD-binding homology is not unexpected. Attached to this review is the alignment scheme for the homology determination between GOX and the D-alanine dehydrogenase from *E. coli*.

DISCLOSURE OF REGISTRATION DATA EXCERPTS APPEARING AS PAGES 11A AND 11B IS SUBJECT TO THE ACCESS PROVISIONS OF FIFRA SECTION 10(g) AS IMPLEMENTED BY THE AFFIRMATION OF NON-MULTINATIONAL STATUS

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD JLK

Secondary Reviewer: Sheryl K. Reilly, Ph.D., Biologist, BPPD SKR

STUDY TYPE:	Product Characterization
MRID NO:	439037-03
CHEMICAL NO:	006401
TEST MATERIAL:	GOX expressed in *E. coli*
STUDY NO:	MSL-12829
SPONSOR:	Monsanto Co., St.Louis, MO
TESTING FACILITY:	Monsanto Co., St. Louis, MO
TITLE OF REPORT:	Production of Glyphosate Oxidoreductase (GOX) in Recombinant *E. coli*
AUTHOR:	Bruce F. Bishop & Mark E. Gustafson
STUDY COMPLETED:	July 1993
CONCLUSION:	The large scale production of an *E. coli* strain expressing the GOX enzyme is described.
CLASSIFICATION:	None assigned. This information is not necessary for risk assessment.

STUDY DESIGN

E. coli strain JM101 was used to express two versions of the GOX protein (GOX(M4-C1) and GOXv247(M4-C1)) as well as a negative control which did not express any protein other than those normally associated with an *E. coli* fermentation. The bacterial cultures were grown in 15 liter Braun fermentors at 37°C with glucose feed, aeration to maintain critical O_2 concentrations and agitation. At an optical density reading of 22± 2, the cultures were induced with nalidixic acid to promote production of the GOX constructs. None of the cultures showed contamination upon culturing at final harvest. All three cultures exhibited similar growth characteristics and yielded about 550 to 720gm of wet cell paste after centrifugation to remove the spent medium. This cell paste was further purified for use in the toxicology and product characterization studies.

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD JLK

Secondary Reviewer: Sheryl K. Reilly, Ph.D., Biologist, BPPD SKR

STUDY TYPE:	Product Characterization
MRID NO:	439037-04
CHEMICAL NO:	006401
TEST MATERIAL:	GOX expressed in *E. coli*
STUDY NO:	93-02-30-01
SPONSOR:	Monsanto Co., St.Louis, MO
TESTING FACILITY:	Monsanto Co., St. Louis, MO
TITLE OF REPORT:	Characterization of Microbially-expressed Proteins: GOX(M4-C1) and GOXv247(M4-C1)
AUTHORS:	Leslie A. Harrison, M.R. Bailey, N.A.Biest, J. Bosse, R.M. Leimgruber, T.E. Nickson & C.E. Smith
STUDY COMPLETED:	April 7, 1994
CONCLUSION:	The two GOX variants, GOX(M4-C1) and GOXv247(M4-C1), expressed in *E. coli* showed similarity to the native GOX protein. These similarities are seen as comparable molecular weights, immunoreactivity, amino acid sequence and enzymatic reactivity.
CLASSIFICATION:	Acceptable.

STUDY DESIGN

The similarity of the *E. coli*-expressed GOX enzyme variants (GOX(M4-C1) from plasmid pMON21117 and GOXv247(M4-C1) from plasmid pMON21115) is compared to a reference of the GOX enzyme (pMON17601) produced in *E. coli*.

TEST MATERIAL

An 80% pure GOX standard (from pMON17061, lot #333-93001) and crude lysates containing the two different GOX variants (GOX(M4-C1), lot #MRB2-22-93 & GOXv247(M4-C1), lot #MRB2-22-93) serve as reference standards. The test substances are GOX(M4-C1), lot #333-93001 & GOXv247(M4-C1), lot #332-93004 both produced from an *E. coli* fermentation. Additional controls included the non-transformed *E. coli*, termed a hollow vector control, and the various buffer systems.

TEST METHODS

The test substances were examined by one-dimensional SDS-PAGE, western blotting, glycosylation staining, N-terminal amino acid sequencing, ELISA and the GOX enzymatic assay described previously (MRID 439037-01).

RESULTS AND DISCUSSION

The apparent molecular weight for the GOX protein was found to be 45,286 daltons for the two test proteins and 44,603 for the reference GOX protein, which was 80% pure. This is in fairly good agreement with the calculated weights of 46,688 for GOX(M4-C1), 46,705 for GOXv247(M4-C1) and 46,124 for the reference GOX standard. The difference between the reference GOX and the test substances is due to the presence of an additional four N-terminal amino acids associated with introducing the chloroplast transit peptide and its protease recognition site. It is important to note that these proteins are expressed in *E. coli* as the chloroplast protease digested fragment, expected to be the active form in the plant itself. The SDS-PAGE gel also showed a band in the hollow vector control, which should not have been present.

The western blot results clarified that the band present in the hollow vector control was not due to the GOX protein but to another protein with the same apparent molecular weight. The western blot showed immunoreactive bands at approximately 45-47kD as expected, but only in the lanes loaded with the test substance or the reference standards. The hollow vector control had no immunoreactive bands. The western blots, which were positive for immunorecognition, were identified by their radioactivity due to binding of the antibody with ^{125}I-labelled protein-G.

Samples were run on SDS-PAGE and stained for the presence of carbohydrate indicating glycoproteins. The results showed positive staining (expressed as an outline, probably due to the amount of protein present) for horseradish peroxidase and ovalbumin controls and negative staining of the non-glycoprotein bands typical for these carbohydrate tests. However, there was also a positively staining band at lower molecular weight in both *E. coli* extracts used to produce GOX.

The amino acid sequence of the first twenty amino acids was that expected for the two test substance proteins. It should be noted

that this sequence is different from that of the native protein, due to the presence of a fragment of the chloroplast transit peptide signal. This fragment alters the second amino acid from a serine to an alanine and adds an additional four amino acids: cysteine, methionine, glutamine and alanine. The sequence was as predicted for 16 of the twenty amino acids for GOX(M4-C1) and eight (+ three probables) of 15 for GOXv247(M4-C1).

The specific activity of the extracts was calculated based on the detected enzyme activity and their protein concentration. To provide a valid comparison, only bacterial extracts producing the standard GOX enzyme were utilized as references. The specific activity of the references and the large scale samples ranged from 3.95 to 8.13 units of activity/mg protein (one unit of GOX activity=1μmole of glyoxylate or AMPA formed per minute).

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD JLK

Secondary Reviewer: Sheryl K. Reilly, Ph.D., Biologist, BPPD SKR

STUDY TYPE:	Product Characterization
MRID NO:	439037-05
CHEMICAL NO:	006401
TEST MATERIAL:	GOX expressed in plants and *E. coli*
STUDY NO:	95-01-39-02
SPONSOR:	Monsanto Co., St.Louis, MO
TESTING FACILITY:	Monsanto Co., St. Louis, MO
TITLE OF REPORT:	Assessment of the Equivalence of GOX Protein Produced in *Escherichia coli* and Insect Protected, Insect protected/Roundup Ready™ and Roundup Ready™ Corn
AUTHOR:	Thomas C. Lee
STUDY COMPLETED:	December 19, 1995
CONCLUSION:	The *E.coli*-expressed GOX protein was shown to have a comparative activity against IDA and glyphosate similar to that expressed in corn line 423-06-1. Corn lines MON 802, 805, 830, 831, 832, 813 and 814 all had detectable levels of immunoreactive bands that co-migrated with the GOX protein in the control extracts.
CLASSIFICATION:	Acceptable.

STUDY DESIGN

The similarity of the GOX enzyme expressed in plants and that produced in *E. coli* and used for product characterization and toxicology is examined by enzymatic activity, molecular weight and immunorecognition.

TEST MATERIALS

The test substances are GOX(M4-C1) as expressed in *E. coli* from plasmid pMON21117 and extracts from several plants expressing the GOX protein. The plant expressing the highest GOX level is a Roundup Ready™ corn line 423-06-1, GOX protein (lot #5752703) was partially purified from this source for the tests. Other plant extracts from field grown corn were made from lines expressing insect protection (MON 809, 810, 813 & 814), glyphosate tolerance (MON 830, 831 & 832) and insect protected/glyphosate tolerance characters (MON 802 & 805). Corn lines (MON 820 & MON 821) with

the same genetic background, but not transformed to express either insect or herbicide resistance, were also included as controls.

TEST METHODS

The extracts were compared using western blot and the GOX assay as described previously (MRID 439037-01).

RESULTS AND DISCUSSION

The enzymatic activity of the bacterial and plant expressed GOX protein (line 423-06-1) show the same preference for iminodiacetic acid (IDA) over glyphosate as seen in the amount of glyoxylate product formed by each substrate and detected by HPLC separation of the 2,4-dinitrophenyl hydrazone derivative. This phenomenon was originally described in the product characterization (MRID 439037-01) and was used here to demonstrate that GOX in either a plant or bacterial expression vehicle continues to behave as expected.

Crude leaf extracts of corn lines MON 802, 805, 810, 830, 831 and 832 were compared to MON 820, 423-06-1 and GOX from *E. coli* on a single western blot. MON 810 and 820 did not show a perceptible GOX band. MON 805 had a weaker band intensity than the other extracts whose bands were all co-migrating and immunoreactive with anti-GOX antibodies. A second western blot was performed which showed lines MON 813 and 814 with faint bands co-migrating with the immunoreactive bands in the positive controls (GOX from *E. coli* and line 423-06-1). Line MON 809 showed no perceptible bands at the GOX location like the negative control line MON 821.

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD JLK

Secondary Reviewer: Sheryl K. Reilly, Ph.D., Biologist, BPPD SKR

STUDY TYPE:	*In vitro* Digestibility
MRID NO:	439037-06
CHEMICAL NO:	006401
TEST MATERIAL:	GOX-enriched protein
STUDY NO:	93-02-30-07
SPONSOR:	Monsanto Co., St.Louis, MO
TESTING FACILITY:	Monsanto Co., St. Louis, MO
TITLE OF REPORT:	Assessment of the *In vitro* Digestive Fate of Glyphosate Oxidoreductase (GOX) and GOXv247 Variant
AUTHORS:	Joel E. Ream, Michele Bailey, Laura Lakemeyer, Mary Taylor, John N. Leach & Thomas E. Nickson
STUDY COMPLETED:	January 28, 1994
CONCLUSION:	Both variants of the GOX protein (GOX(M4-C1) & GOXv247(M4-C1)) are rapidly degraded in simulated gastric fluid (GF) and simulated intestinal fluid (IF). After a fifteen-second incubation in GF, both variants have less than 90% of their initial activity by western blot analysis. Enzyme activity loss is also greater than 90% in both GOX variants when assayed after a one-minute incubation in GF. Similar results are seen in simulated intestinal fluid (IF). Western blot assays show that both variants are greater than 90% degraded by a 30-second incubation in IF. However, the enzyme activity assays show that the GOX(M4-C1) activity lasts longer in IF than variant GOXv247. After a 10-minute IF incubation, the activity decreased to about 48% of initial for GOX(M4-C1), whereas GOXv247 was already greater than 90% inactive.
CLASSIFICATION:	Acceptable.

STUDY DESIGN

A protein extract enriched in GOX protein was subjected to two *in vitro* digestibility assays to determine the stability of this protein to stomach and intestinal fluids.

TEST MATERIALS

The test substances are GOX(M4-C1), lot #333-93001 & GOXv247(M4-C1), lot #332-93004, both produced from an *E. coli* fermentation. The GOX(M4-C1) was produced from plasmid pMON21117 and GOXv247(M4-C1) from plasmid pMON21115. Reference substances for these extracts are GOX purified to greater than 80% (lot #LAH 4-13-92), the hollow vector control and the digestion systems without added protein.

TEST METHODS

The test substances were subjected to simulated digestion using gastric fluid (GF) or intestinal fluid (IF) containing pepsin or pancreatic enzymes, respectively, and prepared according to formulae found in the U.S. Pharmacopeia. The activity of the digestion solutions were verified by digestion of hemoglobin (GF) or resorufin-labelled casein (IF). The enzymatic activity of the fluids was monitored by spectrophotometer for increased absorbance at 280nm (hemoglobin) or 574nm (resorufin-labelled casein) following TCA precipitation.

The test substances were added to the digestion fluids to yield a final concentration of approximately 80µg of GOX or GOXv247 per ml of digestion fluid. The solutions were maintained at 37°C in a shaking water bath and samples removed at the indicated time intervals, except for the intervals less than 2 minutes which were agitated manually. The GF digestions were stopped by the addition of 15µl 0.2M sodium carbonate (pH 11.5) to the 50µl digestion samples. SIF digestions were stopped by heating to 100°C for the western blot assays or by addition of 10µl sample into 10X GOX assay buffer for the GOX enzyme activity assays.

Digestion samples were assayed for active GOX enzyme by both western blot and GOX-specific activity against IDA, as described previously (MRID 439037-01). In order to control for bias, three replicate digestions were run for each treatment. To determine if the samples removed were representative, the whole digestion was quenched and run on SDS-PAGE and compared to the 50µl samples. To verify a lack of interference from other components, a sample digestion with an *E. coli* extract lacking the GOX protein was run through the western blot assay.

RESULTS AND DISCUSSION

Both variants of the GOX protein (GOX(M4-C1) & GOXv247(M4-C1)) are rapidly degraded in simulated gastric fluid (GF) and simulated intestinal fluid (IF). After a fifteen-second incubation in GF, both variants have less than 90% of their initial activity by western blot analysis. Enzyme activity loss is also greater than 90% in both GOX variants when assayed after a one-minute incubation in GF. Similar results are seen in simulated intestinal fluid (IF). Western blot assays show that both variants are greater than 90% degraded by a 30-second incubation in IF. However, the enzyme activity assays show that the GOX(M4-C1) activity lasts longer in IF than variant GOXv247. After a 10-minute IF incubation, the activity decreased to about 48% of initial for GOX(M4-C1), whereas GOXv247 was already greater than 90% inactive.

BPPD COMMENT

It is important to note the results of the western blot assay do not accurately predict the actual functionality of the enzyme being degraded. The company has suggested that the substitution of a histidine residue for an arginine at residue 334 in GOXv247 increased its susceptibility to trypsin attack.

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD JLK

Secondary Reviewer: Sheryl K. Reilly, Ph.D., Biologist, BPPD SKR

STUDY TYPE:	Test Material Characterization
MRID NO:	439037-07
CHEMICAL NO:	006401
TEST MATERIAL:	GOX-enriched protein
STUDY NO:	93-02-30-03
SPONSOR:	Monsanto Co., St.Louis, MO
TESTING FACILITY:	Monsanto Co., St. Louis, MO
TITLE OF REPORT:	Preparation and Confirmation of Doses for Acute Oral Toxicity Studies in Mice with Glyphosate Oxidoreductase GOX(M4-C1) and GOX v247(M4-C1)
AUTHORS:	Thomas E. Nickson, Michele Bailey, Leslie A. Harrison & Jerry Bosse
STUDY COMPLETED:	April 7, 1994
CONCLUSION:	The highest dose administered approached 100mg/kg bodyweight target for the high dose. The actual values were 91.3mg/kg for GOX(M4-C1) and 104mg/kg for COXv247(M4-C1), qunatified by ELISA.
CLASSIFICATION:	Acceptable.

STUDY DESIGN

The preparation and quantification of the GOX dosing material used in the acute oral toxicity test were examined to verify activity and dose administered.

TEST MATERIAL

The test substances are GOX(M4-C1), lot #333-93001 & GOXv247(M4-C1), lot #332-93004, both produced from an *E. coli* fermentation. The GOX(M4-C1) was produced from plasmid pMON21117 and GOXv247(M4-C1) from plasmid pMON21115. The hollow vector control consists of an *E. coli* strain containing the plasmid pMON21106 which lacks the GOX gene and provides a control for the other components in the test material, which is a partially purified fermentation paste.

TEST METHOD

The level of GOX present in the two extracts used for the acute oral toxicity test were examined by ELISA, western blot and GOX enzyme activity. The actual dose was quantified by ELISA.

RESULTS AND DISCUSSION

The highest dose administered approached the 100mg/kg bodyweight target for the high dose. The actual values were 91.3mg/kg for GOX(M4-C1) and 104mg/kg for COXv247(M4-C1). The dosing material was found by western blot to have a protein band which co-migrated with the purified GOX protein and was recognized by anti-GOX antibody derived from goats (MRID 439037-01). The extracts also had GOX enzymatic activity as measured by the release of glyoxylate from either IDA or glyphosate. The actual level of GOX present in the *E. coli* extracts was determined by an ELISA, which had been standardized against a purified GOX extract. The target for the high dose is based on a calculation of the highest human dietary exposure value associated with the consumption of corn from the TAS Exposure 1, Chronic Dietary Exposure report based on the 1987-88 and 1977-78 USDA surveys. This value was 16.9µg/kg BW day for GOX in corn. The calculated safety factor for the dose administered was claimed to be 5591 or 4909 fold for GOX(M4-C1) and GOXv247(M4-C1), respectively.

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD JLK

Secondary Reviewer: Sheryl K. Reilly, Ph.D., Biologist, BPPD SKR

STUDY TYPE:	Acute Oral Toxicity
MRID NO:	439037-08
CHEMICAL NO:	006401
TEST MATERIAL:	GOX enriched protein
STUDY NO:	Monsanto EHL 93079
SPONSOR:	Monsanto Co., St.Louis, MO
TESTING FACILITY:	Monsanto Co., EHL, St. Louis, MO
TITLE OF REPORT:	Acute Oral Toxicity of GOX(M4-C1) Protein in Albino Mice
AUTHOR:	Mark W. Naylor
STUDY COMPLETED:	February 22, 1994
CONCLUSION:	No animals died during the study. The weights of the mice at eight days after dosing were not significantly different between treatment groups. Clinical signs were not observed in any group during the nine-day observation period prior to sacrifice. Gross necropsy revealed a unilateral enlargement of the submaxillary lymph glands mostly in male mice at a rate of from 1-6 individuals out of ten regardless of treatment. The ovaries of the female mice were found to be enlarged at a rate of 3-4 individuals out of ten mice regardless of treatment. Four females were found with enlarged lymph nodes and one with an enlarged spleen. One female displayed an ovarian cyst. None of these findings at gross necropsy indicate an abnormal treatment related pathology.
CLASSIFICATION:	Acceptable.

STUDY DESIGN

Albino CD-1 mice were given a single dose of GOX enriched protein by gavage and examined for 8 to 9 days for clinical signs of toxicity.

TEST MATERIALS

The test substance was GOX(M4-C1) enriched protein (~20% pure) resulting from an *E. coli* cell paste (lot #333-93001). The dosing

suspension was adjusted to yield a final GOX concentration of 91.3, 7.27 and 0.69 mg/kg bodyweight in the dose administered (MRID 439037-07). The dosing material was adjusted with 50mM sodium carbonate buffer to give a final dosing volume of 33.33ml/kg regardless of dose level. This equalization of the dose volume was done to avoid the complications arising from delivering such a high volume dose which could mask any effects from the test material itself.

The test animals were CD-1 albino mice approximately nine-weeks old, obtained from the Charles River Laboratory in Portage, MI and weighing between 28.4 to 34.9 gm (♂) or 24.1 to 27.7 gm (♀). The animals were randomly divided into groups with 10 individuals/sex/treatment based on their weight and lack of observable symptoms of illness. All animals were identified by an ear tag, individually caged and given food and water *ad libitum* after dosing.

TEST METHODS

The animals were dosed by gavage with the test material suspended in carbonate buffer. Controls included dosing with buffer alone or with an extract of the *E. coli* production microbe lacking the GOX gene, termed the hollow vector control. The animals were observed daily in the AM and PM for signs of morbidity, mortality and toxicity. Once a week, the mice were weighed and given a detailed examination for signs of toxicity. On days 8 and 9 after dosing, the animals were sacrificed and examined by gross necropsy.

RESULTS AND DISCUSSION

The weights of the mice at eight days after dosing were either stable or fluctuated slightly (±0.46gm mean body weight). None of the fluctuations were significantly different between treatments. The greatest individual weight loss was 2.6gm in a female in the 10.0 mg/kg GOX dose group. There did not appear to be a treatment related correlation to these fluctuations as the weight loss was seen as frequently in the control groups as in the treatments and the response was not dose related. Clinical signs were not observed in any group during the nine-day observation period prior to sacrifice.

Gross necropsy revealed a unilateral enlargement of the submaxillary lymph glands mostly in male mice at a rate of from 1-6

individuals out of ten regardless of treatment. Three other male mice (no greater than one out of ten per treatment) showed lymph node enlargement in the GOX treated animals. The ovaries of the female mice were found to be enlarged at a rate of 3-4 individuals out of ten mice regardless of treatment. Four females were found with enlarged lymph nodes and one with an enlarged spleen. One female displayed an ovarian cyst. None of these findings at gross necropsy indicate an abnormal, treatment-related pathology. The company suggests that the unilateral inflammation of the submaxillary lymph glands is related to the ear-tagging process although it was not as uniformly apparent in the female mice. No explanation was given for the reported uterine enlargement, which also occurred across all treatments.

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD JLK

Secondary Reviewer: Sheryl K. Reilly, Ph.D., Biologist, BPPD SKR

STUDY TYPE:	Product Characterization
MRID NO:	439037-09
CHEMICAL NO;	006401
TEST MATERIAL:	GOX amino acid sequence
STUDY NO:	MSL-14175
SPONSOR:	Monsanto Co., St.Louis, MO
TESTING FACILITY:	Monsanto Co., St. Louis, MO
TITLE OF REPORT:	Glyphosate Oxidoreductase (GOX) Shares No Significant Sequence Similarity with Proteins Associated with Allergy or Coeliac Disease
AUTHOR:	James D. Astwood
STUDY COMPLETED:	April 17, 1995
CONCLUSION:	None of the amino acid sequences of known allergens or proteins involved in coeliac disease were shown to have similarity to the GOX protein as defined by eight identical and contiguous amino acids in a sequence.
CLASSIFICATION:	Acceptable.

STUDY DESIGN

The amino acid sequence of the GOX protein was compared to other amino acid sequences in a database of known allergen or coeliac disease involved proteins.

STUDY METHODS

A database of known allergens was generated by scanning the GenBank/EMBL ver. 86.0 using the keyword "allergen". The database was improved by removing redundant sequences and adding keywords known to be allergens that were not revealed in the initial search. The sequences that were available only as DNA sequences were translated and entered into the database. This database was scanned using the GOX sequence, with both identical and similar amino acids being identified using the FASTA ver. 1.6. According to the current understanding of allergen epitope structure, sequence similarity to a known allergen was defined as eight identical and contiguous amino acids in a sequence.

RESULTS AND DISCUSSION

None of the amino acid sequences of known allergens or proteins involved in coeliac disease were shown to have similarity to the GOX protein as defined by eight identical and contiguous amino acids in a sequence.

BPPD COMMENTS

While this improved database may be an approach to addressing potential allergenicity in a protein, there is no indication that the supposed eight amino acid sequence homology with known allergens will predict allergenicity. The introduction of such an eight amino acid sequence into a non-allergenic protein and its conversion into an allergen has never been demonstrated.