Early Food Safety Evaluation for a Red Fluorescent Protein: DsRed2

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Key to Abbreviations

~	approximately
DNA	deoxyribonucleic acid
DsRed	the original *Discosoma* sp. red fluorescent protein
DsRed2	variant 2 of the DsRed protein
E. coli	*Escherichia coli*
E score	expectation score
FARRP	Food Allergy Research and Resource Program
GFP	green fluorescent protein
IgE	Immunoglobulin E
ILSI	International Life Sciences Institute
kDa	kilodalton
NCBI	National Center for Biotechnology Information
mM	millimolar
RFP	red fluorescent protein
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGF	simulated gastric fluid
µg	microgram

Abbreviations of units of measurement and of physical and chemical quantities are presented according to the standard format described in Instructions to Authors in the Journal of Biological Chemistry (http://www.jbc.org/).

1. Name, Description and Function of the DsRed2 Protein

The DsRed2 protein (*Discosoma* sp. red fluorescent protein variant 2) belongs to a family of red fluorescent proteins (RFPs) which are members of a group of fluorescent proteins that have been identified in several Anthozoa species (this class of species includes corals, anemones and sea pens). The unique feature of fluorescent proteins is their ability to enter an excited state and emit light of a certain wavelength (*i.e.* fluoresce) upon absorption of ultraviolet or visible light. In nature, fluorescent proteins are thought to provide a photobiological system for regulating the light environment of the host tissues (Schlichter *et al.*, 1986; Salih *et al.*, 2000).

The fluorescent capacity of RFPs is widely exploited in experimental and applied biology. RFPs are used as noninvasive vital color markers for a variety of applications including *in vivo* imaging and monitoring transgene movement in the environment. The utility of the DsRed marker (the original DsRed2 protein) and other DsRed variants was successfully demonstrated in plants in both monocotyledon and dicotyledon species (Jach *et al.*, 2001; Dietrich and Maiss, 2002; Wenck *et al.*, 2003; Mirabella *et al.*, 2004; Stuitje *et al.*, 2003). Notably, plants stably transformed with DsRed did not show any abnormalities, indicating that the protein did not interfere with plant growth, development, fertility, germination or morphogenesis (Jach *et al.*, 2001; Wenck *et al.*, 2003).

2. Description of the Intended Effect of the DsRed2 Protein

Application of the DsRed protein as a visual selection marker for high-throughput seed sorting has been previously demonstrated in *Arabidopsis* (Stuitje *et al.*, 2003). The DsRed2 protein provides a very effective means of tracking transgenic maize seed expressing this protein and is used as a quality assurance color marker that allows monitoring of seeds to ensure seed purity. DsRed2 is a protein with high fluorescent intensity, and its expression, under the control of a seed-preferred promoter, causes red coloration of seed that can be visible to the naked eye. However, to ensure the highest level of quality control and seed purity, detection devices utilizing a fluorescent assay principle are used for automatic identification and sorting of the red seeds. Specifically, the DsRed2 protein will be used as part of a particular inbred seed production process in which seeds containing the fluorescent protein need to be identified and removed.

Standard corn seed production practices involve two sequential steps: an inbred seed production phase and a hybrid seed production phase. In order to ensure successful hybrid production as well as to protect the Company's proprietary germplasm, it is critical that the inbred parent seed (female and male) be genetically pure and securely maintained. Inbred seed production involves growing small quantities of seed in contained, isolated fields to produce quantities of proprietary female and male parent seed. The male and female inbred parent seed are then crossed under controlled conditions to produce hybrid seed. Hybrid seed production requires larger fields that can produce large quantities of hybrid seed which can then be sold to growers.

Presence of the DsRed2 protein screenable marker enables rapid and sensitive detection and efficient removal of the unwanted red fluorescent seeds during inbred seed production. Only seeds that do not contain the DsRed2 protein will be used in the subsequent hybrid seed production. This means the DsRed2 protein will only be present during the small scale contained inbred seed production phase and will then be removed. The DsRed2 protein is not expected to be present in the inbred parents planted for hybrid seed production and so hybrid seed sold to growers will not contain any DsRed2 protein, nor will the grain they produce. Consequently, the DsRed2 protein is not expected to enter the food/feed and grain channels and any exposure to the DsRed2 protein is, therefore, extremely unlikely.

3. Identity and Source of Introduced Genetic Material

The DsRed2 red fluorescent protein (NCBI, GenBank accession number: AAV73970) is a modified variant of the original DsRed protein isolated from a coral-like anemone *Discosoma* sp. (Matz *et al.*, 1999; NCBI, Genbank accession number: AAF03369). Specifically, it was modified to improve the protein's solubility and increase the sensitivity of detection. The DsRed2 protein is 97% identical (6 amino acids differences) to the original DsRed protein isolated from *Discosoma* sp.

Figure 1 represents the amino acid sequence of the DsRed2 protein. The DsRed2 protein is 225 amino acids in length and has an approximate molecular weight of 26 kDa.

Figure 1. Amino Acid Sequence of the DsRed2 Protein

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  1    MASSENVITE FMRFKVRMEG TVNGHEFEIE GEGEGRPYEG HNTVKLKVTK
 51    GGPLPFAWDI LSPQFQYGSK VYVKHPADIP DYKKLSFPEG FKWERVMNFE
101    DGGVATVTQD SSLQDGCFIY KVKFIGVNFP SDGPVMQKKT MGWEASTERL
151    YPRDGVLKGE THKALKLKDG GHYLVEFKSI YMAKKPVQLP GYYYVDAKLD
201    ITSHNEDYTI VEQYERTEGR HHLFL
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The DNA sequence encoding the DsRed2 protein is derived from the original RFP cDNA isolated from a coral-like anemone *Discosoma* sp. (taxonomic classification: Eukaryota; Metazoa; Eumetazoa; Cnidaria; Anthozoa; Zoantharia; Corallimorpharia; Discosomatidae; Discosoma). It is a brightly fluorescent marine organism from the Indo-Pacific area (Matz *et al*., 1999). The *Discosoma* genus has been propagated extensively in captivity as it is an excellent candidate for aquaculture and is commonly used by aquarium hobbyists.

4. Assessment of Allergenicity Potential of the DsRed2 Protein

No single factor has been recognized as the primary indicator for allergenic potential, and no validated animal model that is predictive of allergenic potential is available. Therefore, a step-wise, weight-of-evidence approach was applied to evaluate the allergenic potential of the DsRed2 protein using guidance provided by Codex (2003). This approach takes into account a variety of relevant factors and experimental observations used to derive an overall assessment of the allergenic potential of a protein. Such assessments are typically based on the general properties of food allergens, including the history of exposure and safety of the gene(s) source; bioinformatics comparison of the amino acid sequence of the protein with known protein allergen sequences; physicochemical properties such as stability to pepsin digestion *in vitro* (Thomas *et al.,* 2004), and an estimate of the exposure to the novel protein.

The allergenic potential of the DsRed2 protein was assessed by: 1) bioinformatic comparison of the amino acid sequence of the DsRed2 protein with known or putative protein allergen sequences; 2) evaluation of the stability of the DsRed2 protein using an *in vitro* gastric digestion model; and 3) assessment of the DsRed2 gene source and history of use and exposure.

4.1. Amino Acid Sequence Homology of the DsRed2 Protein to Known Protein Allergens

Bioinformatic analyses were conducted to evaluate the potential allergenicity of the DsRed2 protein. The amino acid sequence of the DsRed2 protein was compared to the Food Allergy Research and Resource Program (FARRP) database version 6 (January 2006; http://www.allergenonline.com/about.asp) containing the amino acid sequences of known or putative allergenic proteins. Potential identities between the DsRed2 protein and proteins in the allergen database were evaluated using the FASTA34 sequence alignment program (Pearson

and Lipman, 1988) set to the default parameters (word size = 2, scoring matrix = BLOSUM50, gap creation penalty = -10, gap extension penalty = -2, E score cutoff = 10). The top scoring alignments were returned and reviewed for identities greater than or equal to 35% over 80 or greater amino acid residues.

None of the alignments met or exceeded the 35% threshold. In addition, the DsRed2 protein was evaluated for any eight or greater contiguous identical amino acid matches to the allergens contained in the database noted above. The use of a match of eight contiguous, identical amino acids appears to have some relevance based upon the reported peptide length for an IgE-binding epitope (Metcalfe *et al*., 1996; Bannon and Ogawa, 2006). Results of the evaluation showed there were no eight or greater contiguous identical amino acid matches observed with the DsRed2 protein. These data indicate the lack of both amino acid identity and immunologically relevant similarities between the DsRed2 protein and known or putative protein allergens.

4.2. Lability of the DsRed2 Protein to Pepsin in Simulated Gastric Fluid (SGF)

A factor that may increase the likelihood of allergic sensitization to proteins via the oral route is the stability of the protein to gastro-intestinal digestion. Proteins that are rapidly digested could be expected to have less opportunity to exert adverse health effects when consumed. The ability of food allergens to remain stable long enough to cross the mucosal membrane of the intestinal tract where absorption occurs is important in the context of a weight-of-evidence approach to understanding a protein's potential allergenic risk (Metcalfe *et al.,* 1996; FAO/WHO, 2001; Codex, 2003; Thomas *et al*., 2004).

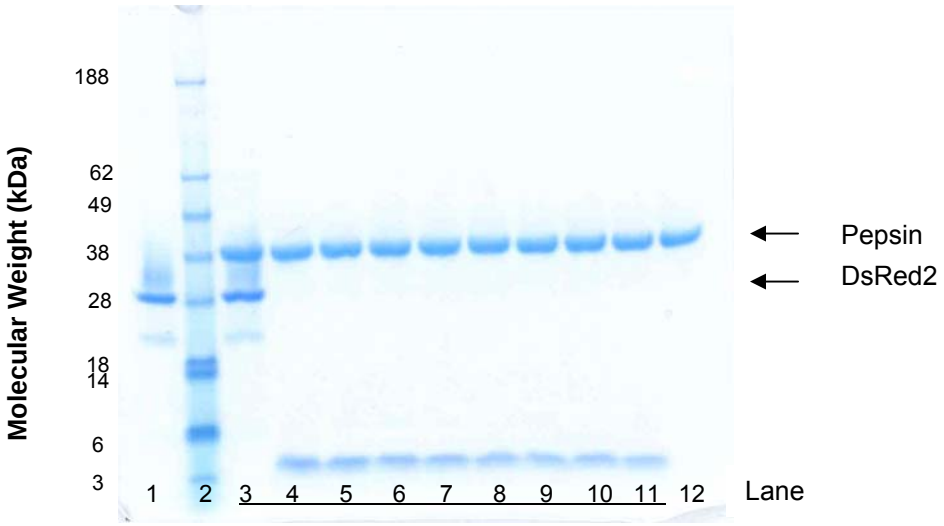
Simulated mammalian gastric fluid (SGF) was used to assess the susceptibility of the DsRed2 protein to proteolytic digestion by pepsin *in vitro*. The International Life Sciences Institute (ILSI) has standardized the pepsin digestibility assay protocol in a multi-laboratory evaluation (Thomas *et al.*, 2004). The SGF formulation, time course, and experimental parameters followed in the evaluation of DsRed2 were similar to conditions used in the ILSI multi-laboratory evaluation.

Bovine serum albumin (BSA) was used as a positive control for this study as it is known to hydrolyze readily in pepsin, and β-lactoglobulin was used as a negative control since it is known to be resistant to pepsin hydrolysis (data not shown). The DsRed2 protein was produced in and purified from a heterologous *E. coli* protein expression system. This protein is equivalent to the DsRed2 protein produced *in planta*. The DsRed2, BSA and β-lactoglobulin proteins were incubated in SGF containing pepsin at pH 1.2 for specific time intervals (0-60 minutes) and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The molar ratio of enzyme to protein in the study was ~0.02 mM pepsin to ~0.01 mM DsRed2, or ~ 2:1. This is equivalent to ~ 3:1 pepsin to DsRed2 ratio on a weight basis (Thomas *et al*., 2004).

The DsRed2 protein was rapidly digested in SGF after less than 0.5 minutes of incubation (Figure 2). A low molecular weight band was visible in all time-course samples of DsRed2 near the dye front and the 3 kDa marker (lanes 4-11). However, results of the bioinformatics search (Section 4.1) demonstrated no significant identity between the amino acid sequence of the DsRed2 protein and known or putative allergenic proteins. These data indicate a lack of immunologically relevant similarities between the DsRed2 protein and know or putative protein allergens. The positive and negative controls, BSA and β-lactoglobulin respectively, were digested as expected.

Results of the SGF study demonstrated that the DsRed2 protein is rapidly (< 30 seconds) hydrolyzed in SGF containing pepsin at pH 1.2 as demonstrated by SDS-PAGE analysis.

Figure 2. Lability of DsRed2 to Pepsin in SGF: Scanned Image of SDS-PAGE Gel



Lane	Sample Identification
1	DsRed2 in water ~60 minutes (2.3 µg)
2	SeeBlue molecular weight marker
3	DsRed2 "Time 0" (2.3 µg)
4	DsRed2 0.5 minutes in SGF
5	DsRed2 1 minute in SGF
6	DsRed2 2 minutes in SGF
7	DsRed2 5 minutes in SGF
8	DsRed2 10 minutes in SGF
9	DsRed2 20 minutes in SGF
10	DsRed2 30 minutes in SGF
11	DsRed2 60 minutes in SGF
12	SGF negative control (no DsRed2) 60 minutes

4.3. DsRed2 Gene Source and History of Exposure

The DsRed2 protein is a modified variant of the RFP originally isolated from a coral-like anemone *Discosoma* sp. (Matz *et al.*, 1999).

Literature available in the public domain was surveyed to identify any publications relating to the DsRed2 protein and more broadly to RFPs. No evidence was found regarding any detrimental effect of the DsRed2 protein or RFPs in general.

As mentioned in Section 1, RFPs have become popular non-invasive vital markers in biology, and are applied in microbiology, plant and animal research. In particular, no detrimental effects of RFPs in regard to growth, development, fertility, germination and morphogenesis have been observed in transgenic plants stably transformed with DsRed, indicating that RFPs do not perturb these plant processes (Jach *et al.*, 2001; Wenck *et al.*, 2003). Although no publicly available data were found that would directly address the safety of the DsRed2 variant of RFP, several publications indirectly address toxicity aspects of other RFP variants. An earlier publication

speculated that the inability to recover transgenic embryonic stem (ES) cell lines could be indicative of possible incompatibility of certain RFPs with normal mouse development (Hadjantonakis *et al.*, 2002). However, this is currently viewed as a likely consequence of obligate tetramer formation *in vivo,* causing deleterious intracellular protein aggregates. Development of new RFP variants with improved solubility and lower tendency to form aggregates in living cells appeared to overcome this problem, as viable transgenic mice lines were successfully generated. High-level ubiquitous expression of mRFP1 (quick-maturing monomeric variant of DsRed) was analyzed in transgenic mice and was concluded not to affect mouse development, general physiology, or reproduction (Zhu *et al.*, 2005). Another improved RFP variant, DsRed.T3, was also successfully expressed in a mouse ES cell line, embryos, and adult animals that also showed no developmental or reproductive abnormalities (Vintersten *et al.*, 2004).

Another body of evidence indicating potential safety of RFPs comes from studies on the more commonly used fluorescent markers, green fluorescent proteins (GFPs) first cloned from Anthozoa species. Although not directly related, DsRed2 is homologous to a green fluorescent protein from the jellyfish *Aequorea victoria* (see Section 5). Various transgenic organisms stably expressing GFP have been successfully generated (Stewart, 2006). Based on an amino acid sequence comparison of GFP to known food allergens and data indicating that GFP was rapidly hydrolyzed in simulated gastric fluid, Richards *et al.* (2003) concluded that “these data indicate that GFP is a low allergenicity risk and provide preliminary indications that GFP is not likely to represent a health risk”. Given structural and functional similarities of GFPs and other Anthozoan fluorescent proteins (*i.e.* RFPs), these fluorescent proteins are not expected to be any different in their oral toxicity and allergenicity (Stewart, 2006).

4.4. Conclusions on Allergenicity Potential of the DsRed2 Protein

Bioinformatic analyses revealed no similarities to known or putative protein allergens for the DsRed2 protein sequence. None of the sequence matches met or exceeded the threshold of greater than or equal to 35% identity over 80 or greater residues. Furthermore, no contiguous stretches of eight or more identical amino acids were shared between the DsRed2 protein and proteins in the allergen database. These data indicate the lack of both amino acid identity and immunologically relevant similarities between the DsRed2 protein and known or putative protein allergens. The DsRed2 protein was rapidly hydrolyzed in simulated gastric fluid. Additionally, although no evidence could be found regarding use of the DsRed2 protein in the food industry, a DsRed variant has been successfully used as a vital marker in mice. Finally, the DsRed2 protein will only be used as part of an internal seed production process and is not expected to be present in any commercial product nor enter the food and feed streams therefore human or animal exposure to this protein will be extremely unlikely. Taken together, these data indicate a lack of allergenic concern for the DsRed2 protein.

5. Assessment of Toxicity Potential of the DsRed2 Protein

The potential toxicity of the DsRed2 protein was assessed by bioinformatic comparison of the amino acid sequence of the DsRed2 protein with publicly available protein sequences. Proteins most similar to the DsRed2 protein were manually inspected to identify any that could be potentially toxic to humans or animals. A close match could be an indicator of toxicological potential of the DsRed2 protein.

A global sequence similarity search of the DsRed2 protein sequence against the NCBI Genbank “nr” dataset was conducted using the BLASTP algorithm. A sequence file comprising the translation of the DsRed2 gene was queried using the BLASTP 2.2.12 algorithm against Release 153.0 (4/15/06) of the “nr” dataset, which incorporates non-redundant entries from all GenBank

nucleotide translations along with protein sequences from SWISS-PROT (http://www.expasy.org/sprot/), PIR (http://pir.georgetown.edu/), PRF (http://www4.prf.or.jp/en/), and PDB (http://www.wwpdb.org/).

One of the most important parameters to monitor when performing similarity searches is the expectation, or *E* score. This *E* score represents the probability that a particular alignment is due to random chance and can be used to evaluate the significance of an alignment. The calculated *E* score depends on the overall length of the aligned sequences (including inserted gaps), the number of identical and conserved residues within the alignment, and the size of the database (Pearson and Lipman, 1988; Baxevanis and Ouellette, 1998). When examining an alignment between two protein sequences, a very low *E* score is more likely to reflect a relevant similarity while a high *E* score (i.e., > 1.0) is more likely to be produced by chance and therefore less biologically relevant.

An *E* score of 1.0 was used to generate biologically meaningful similarity between the DsRed2 protein and proteins in the "nr" dataset. Although a statistically significant sequence similarity generally requires a match with an *E* score of less than 0.01 (Pearson, 2000), a cutoff of $E < 1.0$ insures that proteins with even limited similarity will not be overlooked in the search. Low complexity filtering was turned off and the maximum number of alignments returned was set at 2000.

The DsRed2 protein similarity search identified 491 proteins that were within these criteria. From the 491 returned matches, 490 were to various colored (red, green, yellow, plum, orange, blue) fluorescent proteins from marine anemone (*Discosoma* sp.) and corals (*Acropora*, *Goniopora*, *Montastraea*, *Trachyphyllia*, *Scolymia* and others), as well as numerous synthetic chromoproteins, and one low significance hit to a heme scavenger from *Cyanobacteria*. No matches were observed to known toxins or anti-nutrient proteins.

None of the similar proteins returned by the search were identified as toxins, demonstrating that the DsRed2 protein is unlikely to share relevant sequence similarities with known protein toxins and is therefore unlikely to be a toxin itself.

Additional information supporting the lack of toxicity of the DsRed2 protein is implied by its homology to GFP and by a safety assessment of the GFP. Indeed, when the DsRed2 protein sequence was compared to a GFP from the jellyfish *Aequorea victoria* (NCBI, GenBank accession number: AAB47853), data indicated that the two proteins are homologous. A very low *E* score of e-15 was obtained when the two proteins were compared using a BLAST search, suggesting relevant similarity between the two proteins. In addition, the presence of the GFP domain (pfam01353; CDD41405) was observed in both proteins further suggesting they are related and homologous. The GFP safety assessment study concluded that when fed to rats, GFP was found to be non-toxic when ingested either in a purified form or in diet from transgenic plants (Richards *et al.*, 2003).

Taken together, these data indicate a lack of toxicological concern for the DsRed2 protein.

6. Information on History of Safe Consumption of the DsRed2 Protein in Food

Although no evidence was found regarding the use of the DsRed2 protein in food, an extensive search of literature available in the public domain revealed no information relating to potential risk of adverse effects to human health and the environment.

Additionally, the use of the DsRed2 protein will be solely limited to an internal seed production process. As described in Section 2, the fluorescent protein will be used as a seed marker that will

facilitate identification and removal of these specific seeds. The seed expressing the DsRed2 protein will be strictly contained during this particular inbred seed production process and is not expected to be present during the subsequent phase of hybrid seed production. Therefore, exposure to the DsRed2 protein in food and feed is extremely unlikely.

7. Overall Conclusions

The DsRed2 protein belongs to a family of red fluorescent proteins (RFPs) which are members of a group of fluorescent proteins identified in several Anthozoa species. DsRed2 is a modified variant of the original RFP isolated from a coral-like anemone *Discosoma* sp.

Specifically, the DsRed2 protein will be used as a screenable marker that will enable prompt detection and removal of seed that express the protein. The fluorescent protein will therefore be contained within an internal process and is not intended to be present in a commercial seed product. Therefore, exposure to the DsRed2 protein is extremely unlikely.

Using FDA's recently published guidance for the early food safety evaluation of new proteins in new plant varieties (June 20, 2006: Recommendations for the Early Food Safety Evaluation of New Non-Pesticidal Proteins Produced by New Plant Varieties Intended for Food Use), the DsRed2 protein was evaluated for its allergenicity and toxicity potential.

The allergenic potential of the DsRed2 protein was assessed by: 1) bioinformatic comparison of the amino acid sequence of the DsRed2 protein with known or putative protein allergen sequences; 2) evaluation of the stability of the DsRed2 protein using an *in vitro* gastric digestion model; and 3) assessment of the DsRed2 gene source and history of use or exposure.

Bioinformatic analyses revealed no identities between known or putative protein allergens and the DsRed2 protein sequence. Furthermore, no short (≥ eight amino acids) identical polypeptide matches were shared between the DsRed2 protein and proteins in the allergen database. These data indicate the lack of both amino acid identity and immunologically relevant similarities between the DsRed2 protein and known or putative allergens. The DsRed2 protein was rapidly hydrolyzed in simulated gastric fluid. Additionally, although no evidence could be found regarding the specific use of the DsRed2 protein in the food industry, a DsRed variant has been successfully used as a vital marker in mice. Finally, the DsRed2 protein will only be used as part of an internal seed production process and is not expected to be present in any commercial product. Therefore, exposure to this protein is extremely unlikely. Taken together, these data support the conclusion that the DsRed2 protein is not a potential allergen.

Bioinformatic analyses revealed the DsRed2 protein to be homologous to other colored fluorescent proteins. No biologically relevant sequence similarities were observed between known protein toxins and the DsRed2 protein sequence. Additionally, no evidence of toxicity has been observed in studies that have been conducted using RFPs and GFPs in transgenic plants and animals. These data support the conclusion that the DsRed2 protein is unlikely to be a toxin.

Based on the data and information provided in this submission, we have determined that the DsRed2 protein is unlikely to cause an allergic reaction in humans or be a toxin in humans or animals.

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