

Using LC-MS Based Methods for Testing the Digestibility of a Nonpurified Transgenic Membrane Protein in Simulated Gastric Fluid

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S Supporting Information

ABSTRACT: The digestibility of a nonpurified transgenic membrane protein was determined in pepsin, as part of the food safety evaluation of its resistance to digestion and allergenic potential. Delta-6-desaturase from *Saprolegnia diclina*, a transmembrane protein expressed in safflower for the production of gamma linolenic acid in the seed, could not be obtained in a pure, native form as normally required for this assay. As a novel approach, the endoplasmic reticulum isolated from immature seeds was digested in simulated gastric fluid (SGF) and the degradation of delta-6-desaturase was selectively followed by SDS–PAGE and targeted LC-MS/MS quantification using stable isotope-labeled peptides as internal standards. The digestion of delta-6-desaturase by SGF was shown to be both rapid and complete. Less than 10% of the initial amount of D6D remained intact after 30 s, and no fragments large enough (>3 kDa) to elicit a type I allergic response remained after 60 min.

KEYWORDS: protein digestibility, food safety evaluation, genetically engineered plants, membrane proteins, mass spectrometry, LC-MS

■ INTRODUCTION

Recombinant DNA technologies are increasingly being employed to improve crop yields, disease and insect resistance, herbicide tolerance, nutritional quality, and agronomic performance under adverse environmental conditions. The use of recombinant technologies is considered to be a key to meeting the nutritional demands of an ever increasing population. The safety of new genetically engineered (GE) crops must be established for regulatory approvals, and one aspect of evaluating food safety is determining if a novel protein in GE foods/products has potential to elicit an allergenic response. Food allergens tend to be stable to peptic and acidic conditions of the digestive system, in order to reach and pass through the intestinal mucosa to elicit an allergic response, while non-allergenic food proteins are typically digested relatively quickly.¹ Digestibility by pepsin is included as one of the components of a comprehensive weight-of-evidence approach to assessing allergenic potential of a newly expressed protein,² and the use of in vitro digestions in assessing the food safety of novel proteins is well described.^{3–5}

Here, we report the pepsin digestibility of a transmembrane protein, delta-6-desaturase (D6D), which catalyzes the synthesis of gamma-linolenic acid (GLA) from linoleic acid. A gene from *Saprolegnia diclina* (*S. diclina*) coding for a D6D was engineered into safflower for the production of “high GLA” safflower oil which has many health benefits.^{6,7} The high GLA safflower oil was accepted by the FDA as a new dietary ingredient in 2009 and commercialized as SONOVA 400. In prior work, a targeted proteomics approach showed that *S. diclina* delta-6-desaturase is present in microsomal endoplasmic reticulum (ER) from safflower seeds (Figure S1).⁶ The enzyme level shows a rapid increase during seed development and then

gradually decreases as the seed matures and desiccates until it is no longer detected in mature, dry seeds.

Pepsin digestibility studies of transgenic proteins are normally performed by SDS–PAGE analysis of either purified plant-expressed protein or a recombinant protein expressed by microbes, e.g., *Escherichia coli*, in which the protein has been purified and shown to be equivalent to the protein expressed in crop tissues. Unfortunately, integral membrane proteins such as D6D are very difficult to purify due to their hydrophobic nature and association with lipid bilayers.⁸ Detergents are typically required to disrupt the membrane and maintain solubility, but they can interfere with downstream analyses and potentially denature the protein. However, the use of targeted LC-MS/MS (LC-MS²) permits these intractable proteins to be individually monitored, even in sample preparations containing a complex matrix of other proteins. Here we report the use of targeted mass spectrometry in conjunction with SDS–PAGE which provided the selectivity and sensitivity needed to specifically follow the digestion of D6D among the many other proteins in the ER preparations. These techniques allow not only the detection of minute amounts of a specific protein in the presence of contaminating proteins but also their reliable quantification. Levels of D6D were determined by stable isotope dilution.^{9,10} The use of this method for quantification of membrane proteins was recently reviewed.¹¹ Advancements in mass spectrometry equipment, specialized techniques, and software for protein analysis which have grown tremendously in

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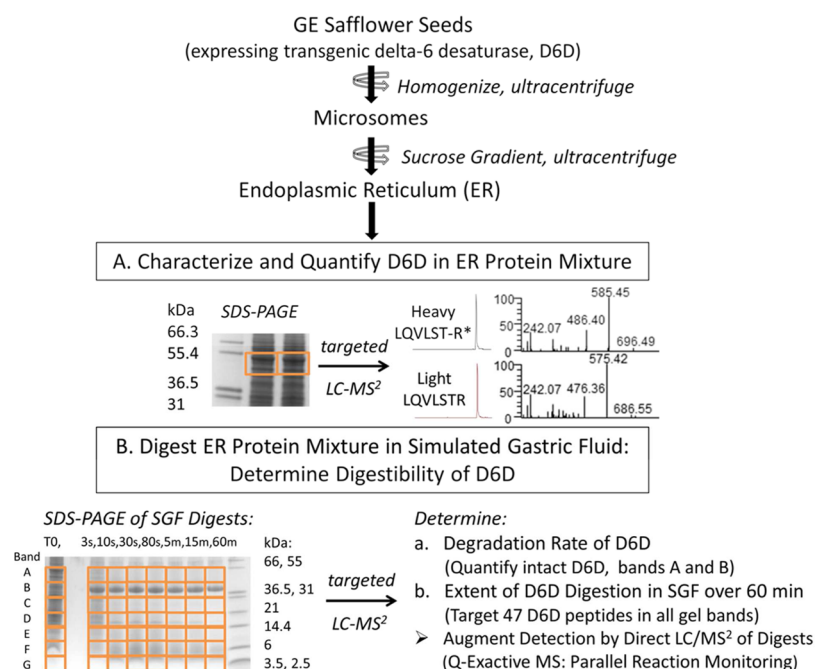


Figure 1. Workflow diagram.

recent years, along with increased availability of lower-cost labeled synthetic peptides, make this approach to protein analysis more accessible than in the past.

MATERIALS AND METHODS

Plant Material. The D6D gene in GLA safflower was sourced from *S. diclina* and is under the control of a seed-specific, *Arabidopsis thaliana*, oleosin promoter so that expression of D6D protein is limited to seeds.⁶ D6D protein was only detectable in immature seeds and with the highest levels in seeds collected 18 to 21 days post anthesis. GLA and control safflower plants were grown in a greenhouse in Woodland, CA, at 18 to 22 °C using natural and artificial light for a 16 h light/8 h dark cycle with watering and fertilization as needed. For this study, immature seeds were collected from several GE and control plants 18 to 21 days post anthesis.

Reagents and Supplies. Porcine pepsin was purchased from Sigma (St. Louis, MO) and sequencing-grade modified trypsin from Promega (Sunnyvale, CA). Stable-isotope (¹³C/¹⁵N) labeled (heavy) peptides specific to D6D were obtained from JPT Peptide Technologies GmbH (Berlin, Germany) and included five purified, quantified SpikeTides TQL (5 nmol each, quantified by amino acid analysis) and 42 crude SpikeTides L (20 μg) peptides. The crude heavy peptides were purified on C18 MicroSpin columns (Nest Group, Southborough, MA) according to the manufacturer's instructions, and individual peptides were characterized by LC-MS and MS² for identity, purity, MS response, and major MS/MS product ions before use. All other chemicals were reagent grade or better.

Protein Extraction and ER Enrichment. A simplified diagram of the workflow is provided as Figure 1. Immature safflower seeds were flash frozen on collection and mechanically ground to a fine powder. D6D protein was extracted from 5 g samples in ice-cold homogenization buffer consisting of 0.3 M Tris-MES, pH 7.6 buffer, 10% glycerol, 0.25 M mannitol, 2 mM EGTA, 1 mM aminocaproic acid, 1 mM benzamidinium HCl, 2.8% PVPP, 1% ascorbic acid, 25 mM potassium metabisulfite, 0.1% Sigma plant protease inhibitor cocktail, and 2 mM DTT by mortar and pestle with sand added. Extracts (~60 mL) were passed through 4 layers of cheese cloth before centrifugation at 12000g for 20 min to remove debris. The supernatant was centrifuged at 100000g for 45 min using a Beckman Optima LE-80K ultracentrifuge and the resultant microsomal pellet resuspended in 0.5 mL of 0.02 M Tris-MES, pH 7.6 buffer, 10% glycerol, 0.25 M

mannitol, 1 mM DTT, and 0.1% plant protease inhibitor cocktail before homogenization with a Potter Elvehjem tissue grinder. Isolation of endoplasmic reticulum (ER) vesicles employed layering microsomal proteins in 5 mL of resuspension buffer onto a 20/34/40% discontinuous sucrose gradient and ultracentrifugation in a swinging bucket rotor at 70000g for 1 h 45 min. ER vesicles were collected from the 20/34% sucrose interface using a Pasteur pipet, diluted at least 5-fold in water, and recentrifuged at 250000g for 30 min before the ER pellet was finally dissolved in a small volume (50 μL) of resuspension buffer without added DTT or plant protease inhibitors. Two batches of ER isolates (101 and 4119) were prepared from separately harvested transgenic GE safflower seeds, and one batch of non-GE control ER was prepared for this study. Total protein concentration was determined using a Qubit 2.0 fluorometer and Qubit Protein Assay Kit (Life Technologies, Carlsbad, CA), and samples were stored at −80 °C.

SDS-PAGE and in Gel Protein Digestion. The ER proteins were denatured and reduced in Laemmli sample buffer plus 10% 2-mercaptoethanol (Bio-Rad, Hercules, CA). Life Technologies Novex 10–20% tricine gel, tricine-SDS running buffer, Mark 12 MW standards, NuPAGE antioxidant, and Colloidal Blue Stain were used per manufacturer's instructions. Gels were imaged with a BioRad ChemiDoc model XRS+ imager with Image Lab software.

Protein bands were excised from the gel using a razor blade and placed on a microscope slide in a biological safety hood. Excised bands corresponding to MW ranges of 47–55, 41–47, 31–41, 21–31, 10–21, 3–10, and 1–3 kDa were diced into approximately 1.5 mm cubes and transferred to 1.5 mL Eppendorf Protein LoBind tubes (Eppendorf, Hamburg, Germany) for further processing and in gel digestion with trypsin. In gel digestion of proteins for subsequent analysis by mass spectrometry has been described previously.¹² Prior to digestion, the diced gel pieces were destained twice with 50% ACN in 25 mM ammonium bicarbonate (AmBic) at 37 °C for 30 min; reduced with 50 mM TCEP in 25 mM AmBic at 60 °C for 10 min; alkylated with 100 mM iodoacetamide in 25 mM AmBic at RT for 60 min; washed with 50% ACN in 25 mM AmBic (37 °C, 15 min); shrunk with excess ACN; and air-dried in a biological safety hood (~20 min).

Processed gel samples were digested with trypsin plus heavy peptide standards at 37 °C overnight. A 2.5 ng/μL trypsin solution in 25 mM AmBic containing 10% ACN provided a protein-to-enzyme ratio of

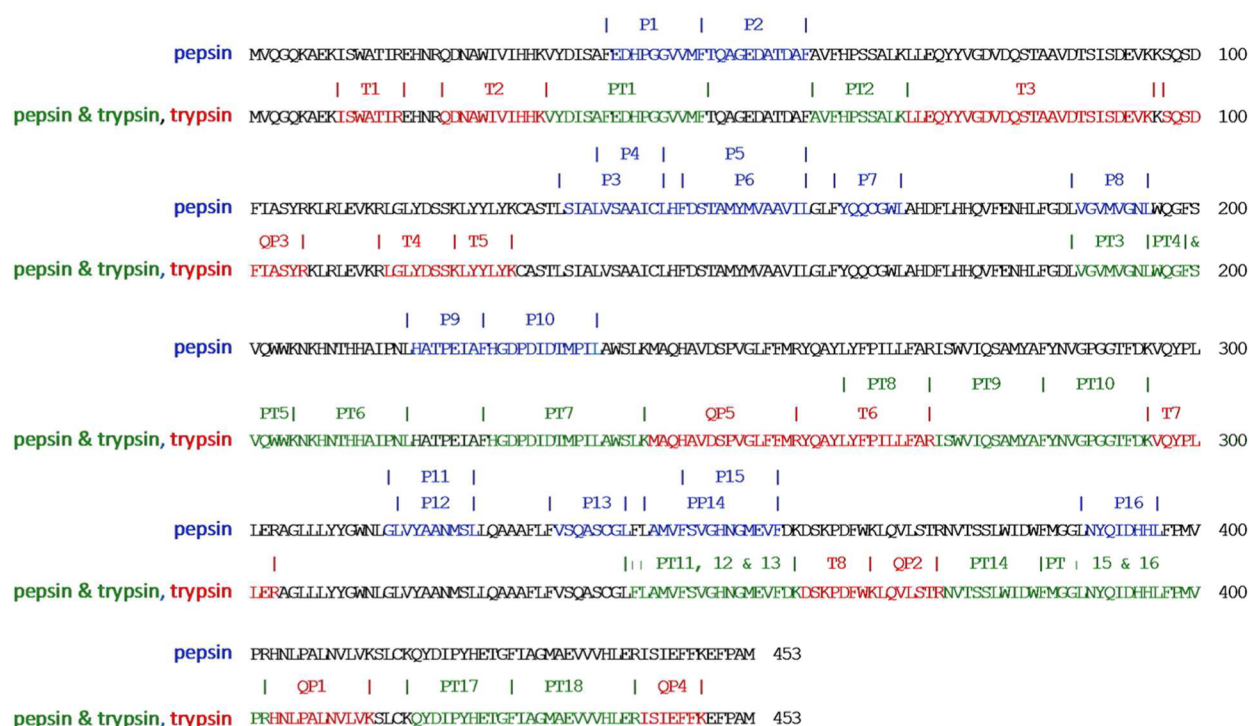


Figure 2. Sequence of D6D protein and location of targeted peptides. Quantitative peptides (QP, underlined); peptides from pepsin cleavage (blue), pepsin followed by trypsin cleavage (PT, green); and peptides from trypsin cleavage (T, red).

approximately 40:1. For quantification of D6D and peptic fragments thereof, the five quantified heavy peptides (2,000 fmol each for quantification of D6D in nondigested ER samples; 4,000 fmol each for detection of fragments after SGF digestion) were mixed with the trypsin solution before its addition to gel pieces and the start of digestion. After digestion, the digest was transferred to autosampler vials (RSA glass, MicroSolv Technologies, Eatontown, NJ) by pipet. Gel pieces were then washed and acidified with 35 μ L of 1.5% formic acid (FA) in water before combining with the harvested digest for LC-MS² analysis.

LC-MS² Quantification. D6D and its fragments were quantified by targeted LC-MS² detection using an LCQ Deca XP+ ion trap mass spectrometer (Thermo Scientific) equipped with a Michrom Captive Spray Source, Paradigm MDLC MS4 HPLC (Bruker-Michrom, Fremont, CA), and HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland). The HPLC was fitted with an ACE C18, 300 Å, 0.3 $\times$ 150 mm capillary column (Advanced Chromatography Technologies, Aberdeen, Scotland) and an Agilent 0.3 $\times$ 5 mm C18 Nano Trap. For injection, a 100 μ L syringe was loaded with 25 μ L of 2% ACN/0.1% FA wash solvent and then 75 μ L of the digest sample to be analyzed. The C18 Nano Trap effluent was diverted to waste during sample loading to desalt the sample before automated valve switching started the analytical run.

Targeted peptides of D6D were eluted from the trap and column using a 5 μ L/min flow rate and a 5 to 40% gradient of ACN in 0.1% FA followed by a 2 min column wash at 80% B in 0.1% FA and a 12 min re-equilibration at 5% ACN in 0.1% FA. For quantification of intact D6D, a 55 min gradient was used in conjunction with the five quantitative peptides while a 90 min gradient was used for detection of all the targeted peptides. Heavy and light (native) peptides were detected using scheduled segments of full-scan MS/MS. Isolation widths of 1.5 or 2.0 Da were used depending on the width required for best trapping efficiencies.

LC-MS² data were processed with Xcalibur 2.2 software using the Quan Browser. The criteria for detection of each native peptide were a distinct peak containing all quantification ions at the same retention time as the corresponding heavy peptide internal standard and with the expected relative ion intensities based on analogous transitions of

the heavy peptide standard. The MS/MS transitions selected for quantification were based on both ion intensities and lack of matrix interference from analysis of nontransgenic control safflower ER samples. As a system suitability control, each sample batch included a gel only control digested along with samples to verify proper operation of the LC-MS² system and detection of spiked heavy peptide internal standards which contain a C-terminal tag that is only removed by trypsin.¹³ The sequence of D6D protein and the positions of targeted quantitative peptides are shown in Figure 2. The quantitative targeted peptides of D6D (QP1–5) were selected based on their prior detection by data-dependent LC/MSⁿ and their lack of post-translational modifications (Figure S1). The amount of D6D protein was calculated on the basis of the isotope dilution method⁹ using the equation (Lt is light peptide and Hv is heavy peptide) as shown for intact D6D below:

fmol of D6D = av peak area ratio:

(LtQP1/HvQP1, LtQP2/HvQP2, LtQP3/HvQP3,

LtQP4/HvQP4, LtQP5/HvQP5)

$\times$ 2000 fmol of heavy peptide added

Protein Digestion in SGF. The digestion of protein test samples in SGF followed established procedures.^{3,14} Two separate preparations of ER isolates containing D6D were digested with SGF in pH 1.2 reaction mixture (0.084 N HCl, 35 mM NaCl, pH 1.2) using 10 units of pepsin per μ g of total protein. D6D enriched ER protein samples and the reaction mixture were prewarmed to 37 °C before the start of digestion. At timed intervals, the digestion was halted by transferring 20 μ L subsamples of the reaction mixture to tubes containing 7 μ L of 200 mM NaHCO₃, pH 11, and 9 μ L of 4 $\times$ Laemmli buffer plus 10% 2-mercaptoethanol. Samples were reduced and denatured prior to SDS–PAGE. For ER batch 101, 8.3 μ g of total protein from each SGF time point was loaded on gel, and for ER batch 4119, 15 μ g of total protein was loaded for each time point.

Controls. To verify pepsin activity and suitability of the SGF digestion methods, protein standards were digested and analyzed by SDS–PAGE. These included soybean trypsin inhibitor (stable to

Table 1. MS/MS Parameters Used for Detection of Quantitative D6D Peptides

peptide	peptide sequence	rt (min)	precursor ion (m/z) ^a	MS/MS quantification ions (m/z)
QP1 (heavy)	HNLPALNVLV-K ^b	35	613.7	861.6, 974.6, 604.5
QP1 (light)	HNLPALNVLVK	35	609.7	853.6, 966.6, 600.5
QP2 (heavy)	LQVLST-R ^b	27	414.1	585.5, 486.3, 242.1
QP2 (light)	LQVLSTR	27	409.1	575.5, 476.3, 242.1
QP3 (heavy)	SQSDFIASY-R ^b	32	592.6	968.5, 583.5
QP3 (light)	SQSDFIASYR	32	587.6	958.5, 578.3
QP4 (heavy)	ISIEFF-K ^b	41	446.6	691.4, 778.4, 578.3
QP4 (light)	ISIEFFK	41	442.6	683.4, 770.4, 570.3
QP5 (heavy)	MAQHAVDSPVGLFFM-R	49	909.3	1178.6, 1277.7, 1063.6, 976.5
QP5 (light)	MAQHAVDSPVGLFFMR	49	904.3	1168.6, 1267.7, 1053.6, 966.5

^aAll are $[M + 2H]^{2+}$ precursor ions. ^bHeavy lysine is U-¹³C6, U-¹⁵N2 (+ 8 Da); heavy arginine is U-¹³C6, U-¹⁵N4 (+ 10 Da).

pepsin), concanavalin A (intermediate stability), and horseradish peroxidase (labile). Protein standards were incubated with 10 units of pepsin per μg of protein in SGF reaction mixture for 0 and 60 min at 37 °C before being quenched and analyzed by SDS–PAGE (2 μg per lane). Assay controls included D6D test sample protein (8.3 μg of ER batch 101) in SGF reaction mixture but without added pepsin; 10% D6D detection controls with quenched pepsin (0.83 μg of ER batch 101); and SGF reaction mixture with pepsin but without the test sample protein. Controls were incubated at 37 °C for 0 and 60 min. Analysis was by SDS–PAGE followed by LC-MS² quantification of intact D6D. These controls were repeated to test the method reproducibility.

ER proteins from immature seeds of nontransgenic parental safflower control (cv. Centennial) were analyzed by SDS–PAGE followed by quantitative LC-MS² of the gel bands corresponding to intact D6D. The control ER proteins were also digested with SGF, separated by SDS–PAGE and the gel zones from several time points (10 s, 30 s, 80 s, and 15 min), then analyzed for potential interferences from digested endogenous proteins using the targeted LC/MS² methods employed for detection of D6D fragments.

Detection of D6D Fragments. Five quantitative and 42 nonquantitative additional peptides were targeted for LC-MS² detection of D6D fragments after digestion of ER samples in SGF. The peptides (Figure 2) were chosen on the basis of predicted peptic and tryptic cleavage sites of D6D in order to provide comprehensive coverage of D6D fragments and to follow their migration to lower MW gel zones as SGF digestion progressed over the 60 min study period. The nonquantitative synthetic heavy peptides were purified, characterized by LC-MS and LC-MS² and normalized in each of the two mixes by combining in proportions according to their individual peak area response on LC/MS. The concentration of heavy peptides in the two mixes was estimated by comparison of their MS responses to the average response of the five quantitative peptides. Due to the large number of peptides, the two separate mixes were used in two separate targeted LC-MS² methods for analysis. The ion-trap MS/MS detection parameters are provided in Table 1 for the quantitative peptides and in Table S1 for the additional peptides.

Direct MS of SGF Digests. Because many of the peptic-only peptides (PP) were not detected in gel samples, additional SGF digests were prepared and analyzed directly without prior SDS–PAGE using a more sensitive and selective mass spectrometer. Transgenic ER protein batch 4119 was digested in SGF as described above for 5 or 10, 30, and 80 s before quenching to pH 7 by addition of pH 11 NaHCO₃. Digests were reduced with 50 mM TCEP, alkylated with 100 mM iodoacetamide, and purified by C18 MicroSpin columns after spiking with heavy peptic peptide standards at $\sim 1,500$ fmol/ μg of total ER protein and then concentrated by SpeedVac (Thermo Scientific, San Jose, CA). Targeted LC-MS² employed a Thermo Q-Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer in parallel reaction monitoring mode (PRM) without multiplexing. A precursor isolation width of 1.6 Da, mass resolution of 17,500 fwhm at 400 m/z , AGC of 1×10^5 , and HCD collision energy of 27% were used for MS/MS. The MS was equipped with an Easy-nLC 1200 delivering a linear gradient

of ACN from 5 to 40% in 0.1% formic acid over 75 min to an EASY-Spray C18 column (0.050 $\times$ 15 cm, 3 μ) at 200 nL/min and EASY-Spray ion source (Thermo Scientific). The mass spectral parameters used for PRM and integration of targeted peptide chromatograms are provided in Table S2.

RESULTS AND DISCUSSION

The use of in vitro SGF digestion to assess the potential of novel proteins to elicit an allergic response is common practice worldwide and was recently reviewed in the literature.¹⁵ These studies typically employ highly purified protein either produced in *E. coli* or isolated from the GE crop itself with analysis by SDS–PAGE. However, membrane proteins are often difficult both to isolate intact from biological material and to produce in *E. coli*.^{8,16} Here, we determined the digestibility of a novel transmembrane protein among other ER membrane proteins using targeted LC-MS². The ER protein test samples were obtained by differential and gradient centrifugation, methods that are routinely used for studying membrane proteins in their native state and which avoids the potential for protein alterations upon more extensive purification. The levels of D6D were determined using targeted LC-MS² and synthetic heavy peptides as internal standards, an approach now being used to monitor protein allergen levels in seeds.¹⁷ The use of targeted LC-MS² detection was instrumental in quantifying and following the digestion of delta-6-desaturase, which represented 0.3% of total protein in the ER protein prepared from immature transgenic safflower seeds. The protein specificity of MS can be superior to Western blots, which are frequently susceptible to nonspecific binding in complex matrices.^{18,19} In addition, MS can allow even single amino acid differences to be distinguished.²⁰

However, targeted LC-MS² is not without its difficulties, and whenever feasible, data dependent acquisition LC-MSⁿ should first be used to aid in selection of highly selective and sensitive proteotypic peptides.²¹ The five quantitative peptide reference standards used here were selected based on their prior detection by data-dependent LC-MS. 42 additional heavy peptides were used to further define the digestion pattern of D6D in SGF by following its fragments throughout different MW zones of the protein gel using LC-MS. These peptides were selected according to those expected from pepsin cleavage of D6D at pH 1.2 (C-terminal to Phe and Leu)²² and those expected from pepsin cleavage followed by trypsin.

Quantification and Characterization of D6D in ER Test Samples. To maximize the concentration of D6D protein present in the extracted safflower samples, immature seeds were harvested between 18 and 21 days. Seed proteins were

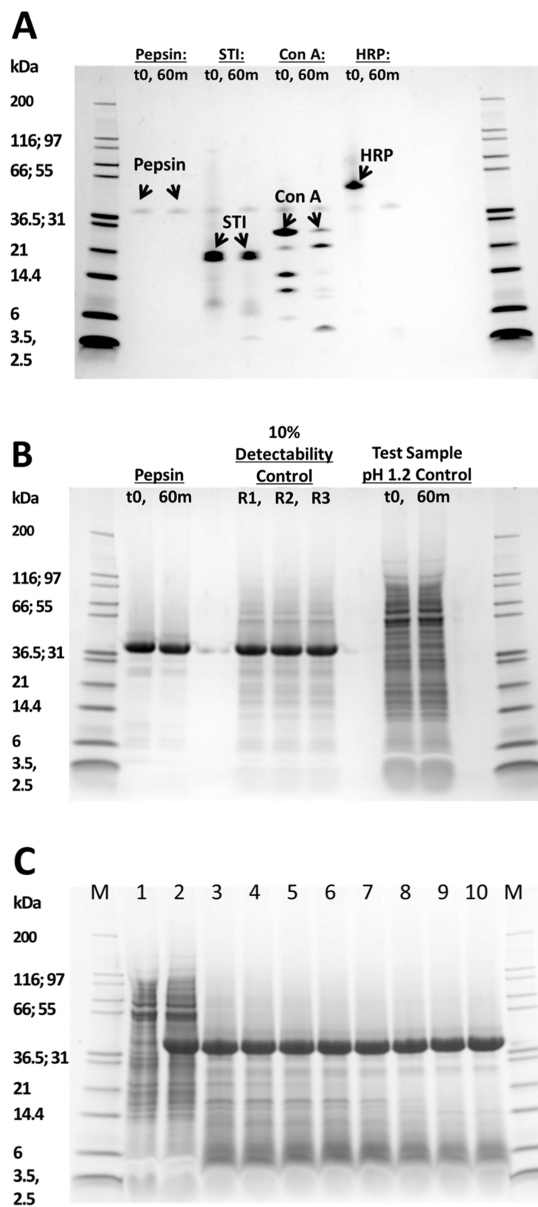


Figure 3. SDS–PAGE of controls and SGF digested ER test sample. (A) Protein standard controls: pepsin, soybean trypsin inhibitor (STI), concanavalin A (Con A), and horseradish peroxidase (HRP) after incubation at 37 °C for 0 and 60 min. (B) Test sample controls: SGF reaction mixture with pepsin (0 and 60 min at 37 °C), 10% detectability controls of D6D test sample, and 100% D6D test sample incubated in SGF reaction mixture without added pepsin (0 and 60 min at 37 °C). (C) SGF digested transgenic ER batch 4119 (15 μ g of total protein/lane): lane M, protein MW markers; lane 1, nondigested test sample without pepsin; lanes 2–9, D6D test sample after digestion in SGF for 0 s, 10 s, 30 s, 60 s, 120 s, 5 min, 15 min, 30 min, and 60 min.

extracted in a homogenization buffer designed for membrane proteins, and the microsomal proteins were prepared by differential centrifugation using standard techniques. D6D protein was further enriched by separation of microsomal proteins on a discontinuous sucrose gradient with collection of the ER fraction at the 20/34% sucrose interface. ER proteins represented ~0.03% of immature seed fresh weight. Two batches of D6D enriched ER protein were characterized and quantified by SDS–PAGE of duplicate 20 μ g samples, followed

Table 2. Controls for the Digestibility Assay of D6D Protein in Simulated Gastric Fluid (SGF)

	control	expt 1 (fmol of D6D)	expt 2 (fmol of D6D)
Detectability Control (10% Level of D6D Test Sample)			
rep 1		43	54
rep 2		44	46
rep 3		44	57
replicate av		44	52
% RSD		0.9%	10%
% accuracy		91%	109%
D6D Test Sample in SGF Reaction Buffer (pH 1.2, 37 °C):			
time 0		485	475
60 min		452	425
% initial D6D after 60 min		93%	89%

by LC–MS². Gel electrophoresis using 10–20% tricine gel and Tris–SDS running buffer provided well-resolved protein bands and MW markers which were used to guide the excision of gel bands containing intact D6D and fragments thereof.

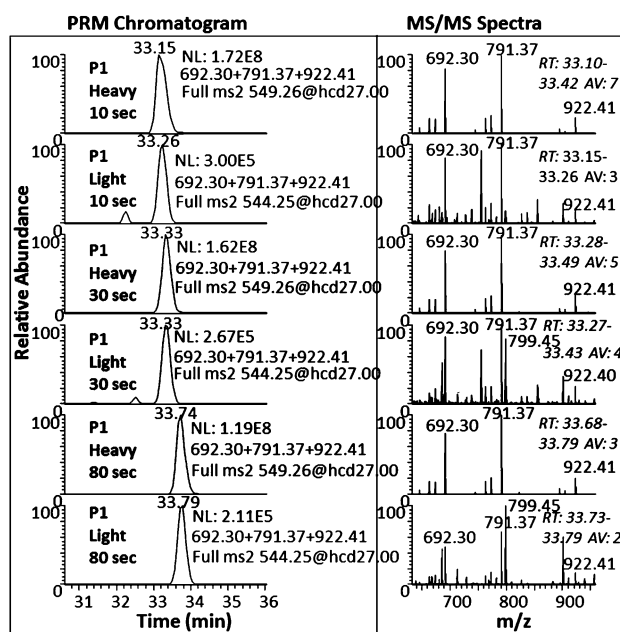
Proteins in excised gel zones were digested in gel with trypsin and the digests then analyzed by LC–MS². Extracted ion chromatograms (EIC) of five quantitative peptides, Table 1, were integrated, and the peak area ratios of light (native) peptides to heavy peptides (added in known amounts) were then used to quantify D6D using the isotope dilution method. Yields of D6D were consistent, in ER batch 101 contained 55 fmol of D6D/ μ g of ER protein (representing 0.28% of total ER protein, w/w) while D6D in batch 4119 contained 63 fmol of D6D/ μ g of ER protein (0.32% of ER protein, w/w). The five quantitative peptides were used in conjunction with other nonquantitative but normalized tryptic peptides (Figure 2) to characterize the D6D protein in test samples by verifying their detection in the gel zones where intact D6D migrates, and these data indicate that the enriched D6D protein remained intact and unaltered by the enrichment procedures (Table S3).

Controls and Method Validation. *Protein Reference Standards.* Protein reference standards were digested in SGF using the same pepsin and ratio of pepsin to protein (10 U/ μ g protein) used for digestion of D6D test protein and analyzed by SDS–PAGE, Figure 3. Soybean trypsin inhibitor (STI) was stable in SGF while concanavalin A was of intermediate stability and horseradish peroxidase was labile. These results were as expected and demonstrated that the SGF digestion conditions used for D6D digestion were appropriate.

Test Sample Controls. Test sample controls included the following: three detectability controls of transgenic ER protein at 10% of the amount used for SGF digestion (ER batch 101); transgenic ER protein incubated in SGF reaction mixture (pH 1.2) without pepsin for 0 and 60 min at 37 °C; and pepsin-only controls incubated in SGF for 0 and 60 min at 37 °C. The analysis of D6D test protein controls by SDS–PAGE, Figure 3, and targeted LC–MS² show that D6D was readily detected at the 10% level with relative standard deviations (RSD) of 10% or less and accuracies of 91 to 109%, Table 2. The reaction buffer controls of D6D in SGF without pepsin indicated that D6D is not substantially degraded at low pH, with between 89 and 93% of D6D remaining intact after 60 min at 37 °C. The test methods were shown to be reproducible with repeated experiments yielding similar results.

Non-GE Safflower Controls. Seeds from nontransgenic parental control safflower were enriched for ER protein alongside transgenic test protein. The control ER protein was

A



B

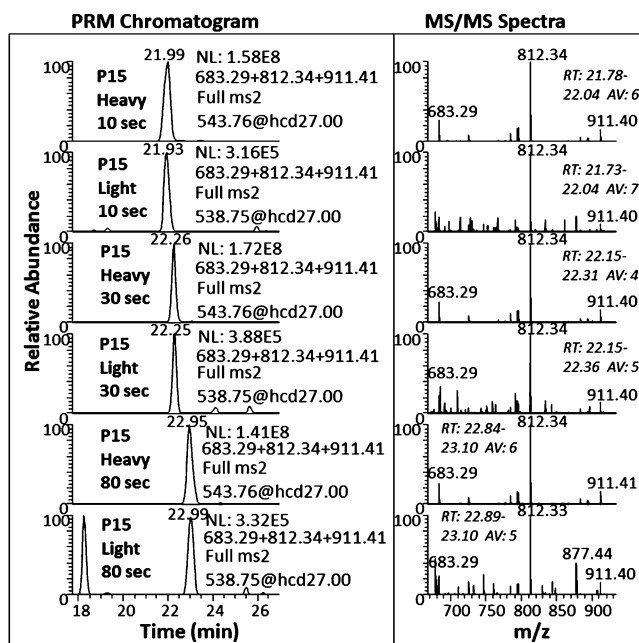


Figure 6. Direct detection of peptic peptides in SGF digests using Q-Exactive MS. (A) Detection of peptide P1 after 10, 30, and 80 s digestions of D6D ER batch 4119 in SGF. (B) Detection of peptide P15 after 10, 30, and 80 s digestions of D6D ER batch 4119 in SGF.

potentially interfering peptide ions. The linearity of the quantification method was established by analysis of pooled D6D gel bands from nondigested ER batch 101 after dilution with pooled D6D gel bands from the nontransgenic control ER. The method was found to be linear throughout the range tested, nominally 4 to 1100 fmol, with an R^2 of 0.999 and with the % of nominal measured ranging from 77 to 104% (Figure S3). The limit of detection (LOD) for ion-trap LC-MS² analysis of D6D was estimated using a signal-to-noise (S/N) ratio of 3:1 with noise determined as the root-mean-square

(Xcalibur software v. 2.2) to be 3 fmol based on the average S/N ratio for light peptides QP1–5 from LC-MS² analysis of the 10% detectability controls. Targeted LC-MS² provided sensitive, accurate, and reproducible results and is suitable for use in protein digestibility studies.

SGF Digestion: Decline of Intact D6D. The digestibility of D6D by SGF was assessed by LC-MS² in conjunction with SDS-PAGE. SDS-PAGE provided separation of intact D6D and fragments thereof, based on size, and provided a means of following the progression of the D6D digestion over time. Intact D6D was found to migrate further than expected based on its MW (51.4 kDa). D6D protein, in nondigested and time 0 samples, was found to be split between the 47–55 kDa and the 41–47 kDa gel zones. Unexpected mobility of membrane proteins on protein gels has been noted previously, presumably due to their hydrophobic nature, tendency to aggregate, and low solubility.^{24,25} Additional excised gel zones included 31–41 kDa, 21–31 kDa, 10–21 kDa, 3–10 kDa, and 1–3 kDa.

Two separate batches of transgenic ER membrane proteins were tested by digestion in SGF with analysis of gel zones containing intact D6D by targeted LC-MS². The degradation of D6D was rapid in both test batches, Figure 5, and demonstrates that the methods used were reproducible. Less than 10% of the starting amount of D6D remained intact after only 30 s, and no intact D6D could be detected after 80 s. The rapid rate of D6D digestion in SGF classifies it as a labile protein that is not resistant to pepsin.

SGF Digestion—Decline of D6D Fragments. MS of Protein Gel Bands. Table 3 shows the distribution of five quantitative peptides over time in gel bands above and below the 3 kDa MW zone. After 5 min in SGF, the most abundant of these peptides (each representing a fragment of D6D) represented only 7% of the T0 level of D6D, and none of these peptides were detected in bands above 3 kDa after 60 min. While these data indicate that the digestion of D6D is complete, 42 additional D6D peptides were targeted for LC-MS² detection using purified, nonquantitative but normalized synthetic heavy peptides as internal standards. These peptides were chosen on the basis of predicted D6D cleavage sites by pepsin at pH 1.2 (C-terminal to F and L)²² and trypsin (C-terminal to R and K) in order to detect fragments of D6D and provide detection coverage over the length of D6D protein, Figure 2. Together, the quantitative and nonquantitative peptides were used to follow the migration of D6D fragments in SDS-PAGE MW zones throughout the 60 min SGF digestion period. The targeted analysis of over 40 D6D peptides by ion-trap MS required splitting the analyses into two separate LC-MS² methods and using a 90 min gradient in order to provide adequate separation and sensitivity. The decline of D6D fragments over time was apparent from the shifting location of peptides detected in the protein gel bands, and a clear pattern of migration to lower MW zones of the SDS-PAGE gel was observed over the 60 min digestion in SGF at 37 °C. These data are presented in Tables S4 and S5. None of the 47 targeted D6D peptides were detected in gel zones above 3 kDa after 60 min.

Direct MS of SGF Digests. The direct analysis of SGF digests without SDS-PAGE was instrumental in detecting some of the smaller peptides produced by pepsin digestion of D6D. These peptides are expected to migrate to the bottom zone (1 to 3 kDa) on SDS-PAGE and, despite extensive fixing of the gel (1 h), could potentially leak out during gel processing and reach levels undetectable by the LCQ Deca XP MS used for gel

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MVQGGKAEKISWATIREHNRQDNWIVIHKKVYDISAFEDHPGGVVMFTQAGEDATDAFAVHPSSALKLLEQYYVGDVDQSTAAVDTSISDEVKKSQSD 100
MVQGGKAEKISWATIREHNRQDNWIVIHKKVYDISAFEDHPGGVVMFTQAGEDATDAFAVHPSSALKLLEQYYVGDVDQSTAAVDTSISDEVKKSQSD 100
FIASYRKLRLVKRLGLYDSSKLYLYKCASTLSIALVSAACILHFDSTAMYMVAAILGLFYQCCGWLADFLHHQVFENHLFGDLVGVVMGNLWQGS 200
FIASYRKLRLVKRLGLYDSSKLYLYKCASTLSIALVSAACILHFDSTAMYMVAAILGLFYQCCGWLADFLHHQVFENHLFGDLVGVVMGNLWQGS 200
VQWKNKHNTHAIPNLHATPEIAFHGDPDIDTMPILAWSLKMAQHAVDSPVGLFFMRYQAYLYFPILLFARISWVIQSAMYAFYNVPGGTFDKVQYPL 300
VQWKNKHNTHAIPNLHATPEIAFHGDPDIDTMPILAWSLKMAQHAVDSPVGLFFMRYQAYLYFPILLFARISWVIQSAMYAFYNVPGGTFDKVQYPL 300
LERAGLLLYGWNILGLVYAANMSLLQAAFLFVSQASCGFLAMVFSVGHNGMEVFDKSKPDFWKLQVLSTRNVTSSLWIDWFMGGLNYQIDHHLFPMV 400
LERAGLLLYGWNILGLVYAANMSLLQAAFLFVSQASCGFLAMVFSVGHNGMEVFDKSKPDFWKLQVLSTRNVTSSLWIDWFMGGLNYQIDHHLFPMV 400
PRHNLPAINVLSLCKQYDIPYHETGFIAGMAEVVHLERISIEFFKEFPAM 453
PRHNLPAINVLSLCKQYDIPYHETGFIAGMAEVVHLERISIEFFKEFPAM 453

```

Figure 7. Protein sequence of D6D showing all peptides detected by targeted LC-MS² after SGF digestion (quantitative peptides are underlined).

samples. However, many of these peptides were detected using parallel reaction monitoring (PRM) on a more sensitive and high mass accuracy instrument, the Q-Exactive plus MS²⁶ (Table S2), and without prior gel electrophoresis. Representative PRM chromatograms and MS² spectra are shown in Figure 6 and Figure S4. The direct detection of peptic peptides P1, 2, 3, 4, 5, 7, 8, 9, 10, 12, 13, 15, and 16 augments the case for exhaustive digestion of D6D in SGF and provides additional details on the specific cleavage points for D6D protein in SGF. Estimated amounts of the identified peptic peptides (from normalization of peak area responses to known amounts of the quantitative peptides) ranged from 7 to 50 fmol per 15 μ g of total ER protein. A fast-scanning, high-resolution, accurate mass MS such as the Q-Exactive can provide the sensitivity and selectivity needed to follow the digestibility of novel proteins in crude preparations without the need for peptide prepreparation by SDS–PAGE, and greatly assist in meeting the challenges of studying membrane proteins.

The allergenic potential of novel proteins is assessed on the basis of both the rate of decline of intact protein in SGF and the completeness of digestion, with protein fragments below 3 kDa in size considered incapable of eliciting a type I allergic response. Type I allergy is an IgE-mediated hypersensitivity disease that is common worldwide. A prerequisite to the release of inflammatory response mediators in type I allergies is the simultaneous binding of IgE Abs to at least two different epitopes on allergens.^{27,28} The presence of at least two different epitopes on a peptide below 3,000 MW (~25 to 30 AA) is unlikely, and the risk of an allergic response is greatly diminished. The sequence of D6D protein showing all of the peptides detected by targeted LC-MS² after SGF digestion is provided as Figure 7. Full coverage of the protein was obtained, and these results indicate that pepsin in SGF degrades D6D to fragments that are too small to elicit a type I allergic response.

To the best of our knowledge, this is the first time targeted LC-MS² was used to follow the digestibility of a nonpurified transgenic protein. The digestibility of a nonpurified transmembrane protein in SGF was successfully assessed using targeted LC-MS² in conjunction with SDS–PAGE and by direct LC-MS² analysis of digests. The use of heavy peptide internal standards provided sensitive, accurate, and reproducible protein quantification and detection of protein fragments over the time course of digestion. While Western blots have been used to follow the digestion of a transgenic protein in a crude protein preparation,²⁹ the use of targeted LC-MS² avoids the challenge of producing antibodies specific to membrane proteins and allows detection of defined regions of the protein, e.g., all regions above 3 kDa in size. LC-MS provides a powerful tool in studying membrane proteins that are difficult to isolate

intact and avoids the question of equivalence when a recombinant protein is used. In conclusion, the membrane protein, delta-6-desaturase, was shown to be labile and rapidly degraded in SGF (pepsin) using LC-MS based methods and a nonpurified membrane protein preparation. Less than 10% of the initial D6D protein remained intact after 30 s in SGF. The degradation in SGF was also complete with no fragments of D6D remaining above 3 kDa in size after 60 min. These results, in conjunction with bioinformatics analysis (data not shown) finding no sequence homology to known allergens, indicate that consumption of this novel protein by humans is unlikely to elicit an allergenic response.

■ ASSOCIATED CONTENT

§ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b01829.

MS/MS, SDS–PAGE, and LC-MS² results (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

D6D, delta-6-desaturase; SGF, simulated gastric fluid; ER, endoplasmic reticulum; LC-MS, liquid chromatography/mass spectrometry; LC-MS², LC/MS/MS; EIC, extracted ion chromatogram; PRM, parallel reaction monitoring; AmBic, ammonium bicarbonate; FA, formic acid; fmol, femtomole; m/z, mass to charge ratio

■ REFERENCES

- (1) Astwood, J. D.; Leach, J. N.; Fuchs, R. L. Stability of food allergens to digestion in vitro. *Nat. Biotechnol.* **1996**, *14*, 1269–1273.
- (2) FAO/WHO Food Standard Programme, Appendix III; *Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants*; Codex Alimentarius Commission: Rome, Italy, 2003; pp 47–60.

- (3) Thomas, K.; Aalbers, M.; Bannon, G. A.; Bartels, M.; Dearman, R. J.; Esdaile, D. J.; Fu, T. J.; Glatt, C. M.; Hadfield, N.; Hatzos, C.; Hefle, S.; Heylings, J. R.; Goodman, R. E.; Henry, B.; Herouet, C.; Holsapple, M.; Ladics, G.; Landry, T. D.; MacIntosh, S. C.; Rice, E. A.; Privalle, L. S.; Steiner, H. Y.; Teshima, R.; van Ree, R.; Woolhiser, M.; Zawodny, J. A multi-laboratory evaluation of a common in vitro pepsin digestion assay protocol used in assessing the safety of novel proteins. *Regul. Toxicol. Pharmacol.* **2004**, *39*, 87–98.
- (4) Fermin, G.; Keith, R. C.; Suzuki, J. Y.; Ferreira, S. A.; Gaskill, D. A.; Pitz, K. Y.; Manshardt, R. M.; Gonsalves, D.; Tripathi, S. Allergenicity assessment of the papaya ringspot virus coat protein expressed in transgenic rainbow papaya. *J. Agric. Food Chem.* **2011**, *59*, 10006–10012.
- (5) Ghosh, P.; Roy, A.; Chakraborty, J.; Das, S. Biological safety assessment of mutant variant of *Allium sativum* leaf agglutinin (mASAL), a novel antifungal protein for future transgenic application. *J. Agric. Food Chem.* **2013**, *61*, 11858–11864.
- (6) Nykiforuk, C. L.; Shewmaker, C.; Harry, I.; Yurchenko, O. P.; Zhang, M.; Reed, C.; Oinam, G. S.; Zaplachinski, S.; Fidantsef, A.; Boothe, J. G.; Moloney, M. High level accumulation of gamma linolenic acid (C18:3Δ6,9,12 *cis*) in transgenic safflower (*Carthamus tinctorius*) seeds. *Transgenic Res.* **2012**, *21*, 367–381.
- (7) Kapoor, R.; Nair, H. Gamma Linolenic Acid Oils. In *Bailey's Industrial Oil and Fat Products*, 6th ed.; Shahidi, F., Ed.; John Wiley and Sons Inc.: Hoboken, NJ, 2005; Vol. 3, Chapter 4.
- (8) Bushey, D. F.; Bannon, G. A.; Delaney, B. F.; Graser, G.; Hefford, M.; Jiang, X.; Lee, T. C.; Madduri, K. M.; Pariza, M.; Privalle, L. S. Characteristics and safety assessment of intractable proteins in genetically modified crops. *Regul. Toxicol. Pharmacol.* **2014**, *69*, 154–70.
- (9) Kippen, A.; Cerini, F.; Vadas, L.; Stocklin, R.; Vu, L.; Offord, R.; Rose, K. Development of an isotope dilution assay for precise determination of insulin, C-peptide, and proinsulin levels in non-diabetic and type II diabetic individuals with comparison to immunoassay. *J. Biol. Chem.* **1997**, *272*, 12513–12522.
- (10) Gerber, S.; Rush, J.; Stemman, O.; Kirschner, M.; Gygi, S. Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 6940–6945.
- (11) Trötschel, C.; Poetsch, A. Current approaches and challenges in targeted absolute quantification of membrane proteins. *Proteomics* **2015**, *15*, 915–929.
- (12) Lahm, H.; Langen, H. Mass spectrometry: a tool for the identification of proteins separated by gels. *Electrophoresis* **2000**, *21*, 2105–2114.
- (13) Bronsema, K.; Bischoff, R.; Van de Merbel, N. High-sensitivity LC-MS/MS quantification of peptides and proteins in complex biological samples: the impact of enzymatic digestion and internal standard selection on method performance. *Anal. Chem.* **2013**, *85*, 9528–9535.
- (14) Bannon, G. A.; Goodman, R. E.; Leach, J. N.; Rice, E.; Fuchs, R. L.; Astwood, J. D. Digestive stability in the context of assessing the potential allergenicity of food proteins. *Comments Toxicol.* **2002**, *8*, 271–285.
- (15) Herman, R. A.; Storer, N. P.; Gao, Y. Digestion assays in allergenicity assessment of transgenic proteins. *Environ. Health Perspect.* **2006**, *114*, 1154–1157.
- (16) Schlegel, S.; Hjelm, A.; Baumgarten, T.; Vikström, D.; de Gier, J. W. Bacterial-based membrane protein production. *Biochim. Biophys. Acta, Mol. Cell Res.* **2014**, *1843*, 1739–1749.
- (17) Stevenson, S. E.; Houston, N. L.; Thelen, J. J. Evolution of seed allergen quantification – From antibodies to mass spectrometry. *Regul. Toxicol. Pharmacol.* **2010**, *58*, S36–S41.
- (18) Liebler, D. C.; Zimmerman, L. J. Targeted quantitation of proteins by mass spectrometry. *Biochemistry* **2013**, *52*, 3797–3806.
- (19) Aebersold, R.; Burlingame, A. L.; Bradshaw, R. A. Western blots versus selected reaction monitoring assays: time to turn the tables? *Mol. Cell. Proteomics* **2013**, *12*, 2381–2382.
- (20) Steen, H.; Mann, M. The ABC's (and XYZ's) of peptide sequencing. *Nat. Rev. Mol. Cell Biol.* **2004**, *5*, 699–711.
- (21) Kuster, B.; Schirle, M.; Mallick, P.; Aebersold, R. Scoring proteomes with proteotypic peptide probes. *Nat. Rev. Mol. Cell Biol.* **2005**, *6*, 577–583.
- (22) Keil, B. In *Specificity of proteolysis*; Springer-Verlag: Berlin-Heidelberg-NewYork, 1992; p 130.
- (23) Yocum, A.; Chinnaiyan, A. Current affairs in quantitative targeted proteomics: multiple reaction monitoring-mass spectrometry. *Briefings Funct. Genomics Proteomics* **2009**, *8*, 145–157.
- (24) Schey, K. L.; Grey, A. C.; Nicklay, J. J. Mass spectrometry of membrane proteins: A focus on aquaporins. *Biochemistry* **2013**, *52*, 3807–3817.
- (25) Bullis, B. L.; Li, X.; Rieder, C. V.; Singh, D. N.; Berthiaume, L. G.; Fliegel, L. Properties of the Na⁺/H⁺ exchanger protein, detergent-resistant aggregation and membrane microdistribution. *Eur. J. Biochem.* **2002**, *269*, 4887–4895.
- (26) Gallien, S.; Duriez, E.; Crone, C.; Kellmann, M.; Moehring, T.; Domon, B. Targeted proteomic quantification on quadrupole-orbitrap mass spectrometer. *Mol. Cell. Proteomics* **2012**, *11*, 1709–1723.
- (27) Huby, R. D.; Dearman, R. J.; Kimber, I. Why are some proteins allergens? *Toxicol. Sci.* **2000**, *55*, 235–246.
- (28) Gieras, A.; Focke-Tejkl, M.; Ball, T.; Verdino, P.; Hartl, A.; Thalhamer, J.; Valenta, R. Molecular determinants of allergen-induced effector cell degranulation. *J. Allergy Clin. Immunol.* **2007**, *119*, 384–390.
- (29) Sinagawa-García, S. R.; Rascón-Cruz, Q.; Valdez-Ortiz, A.; Medina-Godoy, S.; Escobar-Gutiérrez, A.; Paredes-López, O. Safety assessment by in vitro digestibility and allergenicity of genetically modified maize with an amaranth 11S globulin. *J. Agric. Food Chem.* **2004**, *52*, 2709–2714.

SUPPORTING INFORMATION:

Using LC-MS Based Methods for Testing the Digestibility of a Non-Purified Membrane Protein in Simulated Gastric Fluid.

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Table S1. Ion-trap MS/MS Parameters Used for the Detection of D6D Peptides in Protein Gel Bands.

Peptide Name	Peptide Sequence	Ret Time	MW	Precursor ion, m/z	MS/MS ions (most to least abundant)
Light PT1	VYDISAFEDHPGGVVMF	60.5	1881.9	942.5	1234.4 y11; 1087.5 y10; 1392.5 y13; 1587.5
Heavy PT1	VYDISAFEDHPGGVVMF*	60.5	1891.9	947.5	1244.4 y11; 1097.5 y10; 1402.5 y13; 1587.5
Light PT2	AVFHPSSALK	23.9	1055.6	529.0	602.4 y6; 739.4 y7; 443.7 y8 2+; 886.5 y8
Heavy PT2	AVFHPSSALK*	23.9	1063.6	533.0	610.4 y6; 747.4 y7, 447.7 y8 2+, 894.5 y8
Light PT3	VGVMVGNLWQGFSVQWWK	77.7	2120.1	1061.3	1351.6 y10; 1037.5 y8; 1635.7 y13; 833.4 y6
Heavy PT3	VGVMVGNLWQGFSVQWWK*	77.7	2128.1	1065.3	1359.6 y10; 1045.5 y8; 1643.7 y13; 841.4 y6
Light PT4	WQGFVQWWK	60.5	1350.7	676.6	1037.5 y8; 833.5 y6; 315.1 b2
Heavy PT4	WQGFVQWWK*	60.5	1358.7	680.6	1045.5 y8; 841.5 y6; 315.1 b2
Light PT5	SVQWWK	39.0	832.4	833.6	501.2 b5; 519.3 y3; 333.2 y2; 629.3 y4-H2O
Heavy PT5	SVQWWK*	39.0	840.4	841.6	501.2 b5; 527.3 y3; 341.2 y2; 637.3 y4-H2O
Light PT6	HNTHHAIPNL	21.4	1152.6	577.3	811.4 b7; 343.2 y3; 698.3 b6
Heavy PT6	HNTHHAIPNL*	21.4	1159.6	580.8	811.4 b7; 350.2 y3; 698.3 b6
Light PT7	HGDPDIDTMPILAWSLK	64.2	1908.0	637.6	927.6 y8; 464.3 y8 2+; 479.0
Heavy PT7	HGDPDIDTMPILAWSLK*	64.2	1916.0	640.3	935.6 y8; 468.3 y8 2+; 483.0
Light PT8	YFPILLFAR	69.9	1138.7	570.5	829.9 y7; 415.4 y7 2+; 311.0 b2
Heavy PT8	YFPILLFAR*	69.9	1148.7	575.5	839.9 y7; 420.4 y7 2+; 311.0 b2
Light PT9	ISWVIQSAMYAF	71.0	1414.7	1415.7	1250.6 b11; 1179.6 b10; 1232.6 b11-H2O
Heavy PT9	ISWVIQSAMYAF*	71.0	1424.7	1425.7	1250.6 b11; 1179.6 b10; 1232.6 b11-H2O
Light PT10	YNVGPGGTFDK	25.7	1153.5	578.1	778.4 y8; 877.4 y9; 721.4 y7; 278.0 b2
Heavy PT10	YNVGPGGTFDK*	25.7	1161.5	582.1	786.4 y8; 885.4 y9; 729.4 y7; 278.0 b2
Light PT11	FLAMVFSVGHNGMEVFDK	64.0	2027.0	1014.8	1133.5 y10; 1466.6 y13; 1232.6 y11; 1319.5
Heavy PT11	FLAMVFSVGHNGMEVFDK*	64.0	2035.0	1018.8	1141.5 y10; 1474.6 y13; 1240.6 y11; 1327.5

K*= Lys U-¹³C6,U-¹⁵N2 ; R*= Arg U-¹³C6,U-¹⁵N4; L*=Leu U-¹³C6,U-¹⁵N; C(CAM)=Carbamoylmethylated Cys.

Table S1 cont. Ion-trap MS/MS Parameters Used for the Detection of D6D Peptides in Protein Gel Bands.

Peptide Name	Peptide Sequence	Ret Time	MW	Precursor ion, m/z	MS/MS ions (most to least abundant)
Light PT12	LAMVFSVGHNNGMEVFDK	54.5	1879.9	941.6	1133.5 y10; 1466.7 y13; 1232.6 y11; 1319.6 y12
Heavy PT12	LAMVFSVGHNNGMEVFDK*	54.5	1887.9	945.6	1141.5 y10; 1474.7 y13; 1240.6 y11; 1327.6 y12
Light PT13	AMVFSVGHNNGMEVFDK	48.0	1766.8	885	1133.5 y10; 1076.5 y9; 733.8 y13 2+; 1359.6 b13
Heavy PT13	AMVFSVGHNNGMEVFDK*	48.0	1774.8	889.0	1141.5 y10; 1084.5 y9; 737.8 y13 2+; 1359.6 b13
Light PT14	NVTSSLWIDWF	78.4	1366.7	1367.7	1202.6 b10; 901.5 b8; 1016.5 b9; 1185.4 b10-17
Heavy PT14	NVTSSLWIDWF*	78.4	1376.7	1377.7	1202.6 b10; 901.5 b8; 1016.5 b9; 1185.4 b10-17
Light PT15	MGGLNYQIDHHLFPMVPR	53.2	2124.0	709.2	599.4 y5; 764.3 b7, 883.9 y14 2+; 997.5 y17 2+
Heavy PT15	MGGLNYQIDHHLFPMVPR*	53.2	2134.0	712.5	609.4 y5; 764.3 b7, 888.9 y14 2+; 1002.5 y17 2+
Light PT16	NYQIDHHLFPMVPR	45.3	1765.9	884.3	599.4 y5; 746.4 y6; 996.6 y8; 1168.5 b9; 1133.5 y9
Heavy PT16	NYQIDHHLFPMVPR*	45.3	1775.9	889.3	609.4 y5; 756.4 y6; 1006.6 y8; 1168.5 b9; 1143.5 y9
Light PT17	QYDIPYHETGF	40.3	1368.6	685.8	850.4 y7; 672.0 b11; 502.2 b4-H2O; 1078.5 y9
Heavy PT17	QYDIPYHETGF*	40.3	1378.6	690.8	860.4 y7; 682.0 b11; 502.2 b4-H2O; 1088.5 y9
Light PT17	pyroGlu QYDIPYHETGF	45.4	1351.6	1352.6	850.4 y7; 1029.5 b8-17; 1334.6 b11-17
Heavy PT17	pyroGlu QYDIPYHETGF*	45.4	1361.6	1362.6	860.4 y7; 1029.5 b8-17; 1344.6 b11-17
Light PT18	IAGMAEVVVHLER	46.3	1422.8	712.6	752.4 y6; 554.4 y4; 653.4 y5; 851.5 y7; 980.5 y8
Heavy PT18	IAGMAEVVVHLER*	46.3	1432.8	717.6	762.4 y6; 564.4 y4; 663.4 y5; 861.5 y7; 990.5 y8
Light T1	ISWATIR	38.9	845.5	424.0	646.5 y5; 460.4 y4; 733.5 y6
Heavy T1	ISWATIR*	38.9	855.5	429.0	656.5 y5; 470.4 y4; 743.5 y6
Light T2	QDNAWIVIIHK	30.2	1359.7	454.2	448.6 b11 3+; 672.5 b11 2+; 534.3 y4
Heavy T2	QDNAWIVIIHK*	30.2	1367.7	457.1	451.3 b11 3+; 676.5 b11 2+; 542.3 y4
Light T3	LLEQYYVGVDVQSTAAVDTSISDEVK	52.9	2844.4	1423.7	993.5 y9; 1163.6 y11; 1936.7 y19
Heavy T3	LLEQYYVGVDVQSTAAVDTSISDEVK*	52.9	2852.4	1427.4	1001.5 y9; 1171.60 y11; 1944.7 y19

K*= Lys U-¹³C6,U-¹⁵N2 ; R*= Arg U-¹³C6,U-¹⁵N4; L*=Leu U-¹³C6,U-¹⁵N); C(CAM)=Carbamoylmethylated Cys.

Table S1 cont. Ion-trap MS/MS Parameters Used for the Detection of D6D Peptides in Protein Gel Bands.

Peptide Name	Peptide Sequence	Ret Time	MW	Precursor ion, m/z	MS/MS ions (most to least abundant)
Light T4	LGLYDSSK	22.0	881.5	442.0	599.3 y5; 712.4 y6; 769.4 y7
Heavy T4	LGLYDSSK*	22.0	889.5	446.0	607.3 y5; 720.4 y6; 777.4 y7
Light T5	LYYLYK	33.4	861.5	862.5	440.1 b3; 553.2 b4
Heavy T5	LYYLYK*	33.4	869.5	870.5	440.1 b3; 553.2 b4
Light T6	YQAYLYFPILLFAR	85.3	1777.0	889.8	1139.7 y9; 976.6 y8; 829.5 y7; 1252.7 y10
Heavy T6	YQAYLYFPILLFAR*	85.3	1787.0	894.8	1149.7 y9; 986.6 y8; 839.5 y7; 1262.7 y10
Light T7	VQYPLLER	38.0	1016.6	509.4	790.4 y6; 627.4 y5; 391.2 b3
Heavy T7	VQYPLLER*	38.0	1026.6	514.4	800.4 y6; 637.4 y5; 391.2 b3
Light T8	DSKPDFWK	28.6	1021.5	512.1	692.3 y5; 454.4 y7 2+; 480.3 y3
Heavy T8	DSKPDFWK*	28.6	1029.5	516.1	700.3 y5; 458.4 y7 2+; 488.3 y3
Light P1	EDHPGGVVMF	37.4	1086.5	544.3	791.4 b8; 922.4 b9; 773.4 b8-H2O
Heavy P1	EDHPGGVVMF*	37.4	1096.5	549.3	791.4 b8; 922.4 b9; 773.4 b8-H2O
Light P2	TQAGEDATDAF	23.6	1124.5	1125.5	942.4 b10-H2O; 960.4 b10; 889.4 b9; 896.4 y9
Heavy P2	TQAGEDATDAF*	23.6	1134.5	1135.5	942.4 b10-H2O; 960.4 b10; 889.4 b9; 906.4 y9
Light P3	SIALVSAAIC(CAM)L	59.9	1116.6	1117.6	808.4 b9-H2O; 826.4 b9; 695.3 b8-H2O; 986.4 b10
Heavy P3	SIALVSAAIC(CAM)L*	59.9	1123.6	1124.6	808.4 b9-H2O; 826.4 b9; 695.3 b8-H2O; 986.4 b10
Light P4	VSAAIC(CAM)L	36.9	733.4	733.4	442.1 b5; 602.2 b6; 722.3 b7
Heavy P4	VSAAIC(CAM)L*	36.9	740.4	740.4	442.1 b5; 602.2 b6; 722.3 b7
Light P5	HFDSTAMYMVAAVIL	75.1	1667.8	835.0	713.4 b13; 766.0 b14; 663.4 b12 (all 2+)
Heavy P5	HFDSTAMYMVAAVIL*	75.1	1674.8	838.4	713.4 b13; 769.5 b14; 663.4 b12 (all 2+)
Light P6	DSTAMYMVAAVIL	64.3	1383.7	1384.6	1253.6 b12; 1140.5 b11, 1041.4 b10
Heavy P6	DSTAMYMVAAVIL*	64.3	1390.7	1391.6	1253.6 b12; 1140.5 b11, 1041.4 b10

K*= Lys U-¹³C6,U-¹⁵N2 ; R*= Arg U-¹³C6,U-¹⁵N4; L*=Leu U-¹³C6,U-¹⁵N); C(CAM)=Carbamoylmethylated Cys.

Table S1 cont. Ion-trap MS/MS parameters used for the detection of D6D peptides in protein gel bands.

Peptide Name	Peptide Sequence	Ret Time	MW	Precursor ion, m/z	MS/MS ions (most to least abundant)
Light P7	YQQC(CAM)GWL	40.3	953.4	954.4	823.3 b6; 806.3 b6-17; 663.3 y5
Heavy P7	YQQC(CAM)GWL*	40.3	960.4	961.4	823.3 b6; 806.3 b6-17; 670.3 y5
Light P8	VGVMVGNL	41.0	787.4	788.4	657.3 b7; 771.3 b8; 486.3 b5; 753.4 b8-H2O
Heavy P8	VGVMVGNL*	41.0	794.4	795.4	657.3 b7; 778.3 b8; 486.3 b5; 760.4 b8-H2O
Light P9	HATPEIAF	34.0	884.4	885.4	867.5 b8; 720.4 b7; 576.3 y5
Heavy P9	HATPEIAF*	34.0	894.4	895.4	877.5 b8; 720.4 b7; 586.3 y5
Light P10	HGDPDIDTMPIL	48.5	1322.6	662.3	982.4 b9; 596.8 b11 2+ (635.3 b6; 851.3 b8)
Heavy P10	HGDPDIDTMPIL*	48.5	1329.6	665.8	982.4 b9; 596.8 b11 2+ (635.3 b6; 851.3 b8)
Light P11	VYDISAFEDHPGGVVMF	60.5	1881.9	942.5	1234.4 y11; 1087.5 y10; 1392.5 y13; 1587.5 b15
Heavy P11	VYDISAFEDHPGGVVMF*	60.5	1891.9	947.5	1244.4 y11; 1097.5 y10; 1402.5 y13; 1587.5 b15
Light P12	VYAANMSL	35.0	867.4	868.4	633.3 b6-17; 650.3 b6; 720.3 b7-17
Heavy P12	VYAANMSL*	35.0	874.4	875.4	633.3 b6-17; 650.3 b6; 720.3 b7-17
Light P13	VSQASC(CAM)GL	13.6	820.4	821.4	633.2 b6; 803.4 b8; 615.2 b6-H2O; 635.3 y6
Heavy P13	VSQASC(CAM)GL*	13.6	827.4	828.4	633.2 b6; 810.4 b8; 615.2 b6-H2O; 642.3 y6 792.3
Light P14	AMVFSVGHNHNGMEVF	53.5	1523.7	763.4	630.8 b12 2+; 680.6 b13 2+; 1260.6 b12
Heavy P14	AMVFSVGHNHNGMEVF*	53.5	1533.7	768.4	630.8 b12 2+; 680.6 b13 2+; 1260.6 b12
Light P15	SVGHNGMEVF	33.3	1075.5	538.8	911.4 b9; 812.4 b8; 456.4 b9 2+
Heavy P15	SVGHNGMEVF*	33.3	1085.5	544.1	911.4 b9; 812.4 b8; 456.4 b9 2+
Light P16	NYQIDHHL	21.6	1038.5	520.3	634.3 y5; 762.4 y6; 511.9 b8 2+; 406.2 b3
Heavy P16	NYQIDHHL*	21.6	1045.5	523.8	641.3 y5; 769.4 y6; 515.4 b8 2+; 406.2 b3

K*= Lys U-¹³C6,U-¹⁵N2 ; R*= Arg U-¹³C6,U-¹⁵N4; L*=Leu U-¹³C6,U-¹⁵N); C(CAM)=Carbamoylmethylated Cys.

Table S2. Q-Exactive™ MS/MS Parameters Used for the Detection of D6D Peptides in SGF Digests, Without Prior SDS-PAGE.

Peptide	Peptide Sequence	Ret Time (min)	Mono-isotopic MW	Precursor mass ^a (m/z)	Ions used for parallel reaction monitoring (m/z) ^b	~ fmol/15 µg ER protein (sec in SGF) ^c
Light P1	EDHPGGVVMF	34	1087.5	544.25	692.30 b7; 791.37 b8; 922.41 b9	21 (10s); 24 (30s); 25 (80s)
Heavy P1	EDHPGGVVMF*	34	1097.5	549.26	692.30 b7; 791.37 b8; 922.41 b9	
Light P2	TQAGEDATDAF	23	1125.5	1125.47	825.33 y8; 896.36 y9; 774.33 b8; 889.35 b9; 960.39 b10	n.a.; n.a.; 10 (80s)
Heavy P2	TQAGEDATDAF*	23	1135.5	1135.50	835.35 y8; 906.38 y9; 774.33 b8; 889.35 b9; 960.39 b10	
Light P3	SIALVSAAIC(CAM)L	56	1116.6	1117.61	272.16 b3; 385.24 b4; 695.41 b8-H ₂ O; 713.42 b8	11 (5s); 65 (30s); 56 (80s)
Heavy P3	SIALVSAAIC(CAM)L*	56	1123.6	1124.61	272.16 b3; 385.24 b4; 695.41 b8-H ₂ O; 713.42 b8	
Light P4	VSAAIC(CAM)L	36	732.4	733.37	292.13 y2; 329.18 b4; 442.27 b5	14 (5s); 14 (30s); 19 (80s)
Heavy P4	VSAAIC(CAM)L*	36	739.4	740.37	299.15 y2; 329.18 b4; 442.27 b5	
Light P5	HFDSTAMYMVAAVIL	67	1667.8	834.91	477.20 b8; 592.25 b10 ; 627.77 b11 -- all are 2+ ions	0 (5s); 6 (30s); 6 (80s)
Heavy P5	HFDSTAMYMVAAVIL*	67	1674.8	838.41	477.20 b8; 592.25 b10 ; 627.77 b11 -- all are 2+ ions	
Light P6	DSTAMYMVAAVIL	--	1383.7	1384.69	not detected	not detected
Heavy P6	DSTAMYMVAAVIL*		1390.7	1391.69		
Light P7	YQQC(CAM)GWL	41	953.4	954.39	292.13 b2; 420.19 b3	20 (5s); 27 (30s); 20 (80s)
Heavy P7	YQQC(CAM)GWL*	41	960.4	961.39	292.13 b2; 420.19 b3	

F*=L-Phenylalanine (¹³C9, ¹⁵N); L*=L-Leucine (¹³C6, ¹⁵N); C(CAM)=Carbamoylmethylated Cysteine; n.a. = not analyzed.

^a Parameters for targeted LC-MS² by PRM: Resolution = 17,500 FWHM at m/z 400; 1.6 Da isolation width; 1 x 105 AGC; HCD CE = 27%; no scheduling.

^b A mass tolerance of 20 ppm was used to create parallel reaction monitoring chromatograms for peptide detection and integration.

^c Approximate fmol based on normalization of individual peptide peak area responses to the average response of QP peptides.

Table S2 cont. Q-Exactive™ MS/MS Parameters Used for the Detection of D6D Peptides in SGF Digests, Without Prior SDS-PAGE.

Peptide	Peptide Sequence	Ret Time (min)	Mono-isotopic MW	Precursor mass ^a (m/z)	Ions used for parallel reaction monitoring (m/z) ^b	~ fmol/15 µg ER protein (sec in SGF) ^c
Light P8	VGVMVGNL	40	787.4	788.43	256.17 b3; 387.21 b4; 486.28 b5	49 (10s); 24 (30s); 20 (80s)
Heavy P8	VGVMVGNL*	40	794.4	795.43	256.17 b3; 387.21 b4; 486.28 b5	
Light P9	HATPEIAF	24	885.5	443.23	166.09 y1; 237.13 y2; 310.15 b3 (209.10 b2)	n.a., n.a., 70 (80s)
Heavy P9	HATPEIAF*	24	895.5	448.24	176.11 y1; 247.15 y2; 310.15 b3 (209.10 b2)	
Light P10	HGDPDIDTMPIL	45	1323.6	662.32	342.24 y3; 982.39 b9; 851.35 b8 (635.3 b6)	10 (10s); 9 (30s); 10 (80s)
Heavy P10	HGDPDIDTMPIL*	45	1330.6	665.82	349.26 y3; 982.39 b9; 851.35 b8 (635.3 b6)	
Light P12	VYAANMSL	33	867.4	868.42	334.18 b3; 650.30 b6 (263.14 b2)	0.5 (10s); 15 (30s); 18 (80s)
Heavy P12	VYAANMSL*	33	874.4	875.44	334.18 b3; 650.30 b6 (263.14 b2)	
Light P13	VSQASGL	12	820.4	821.38	349.15 y3; 436.18 y4 (386.20 b4, 473.2 b5)	11 (10s); 15 (30s); 31 (80s)
Heavy P13	VSQASC(CAM)GL*	12	827.4	828.40	356.18 y3; 443.20 y4 (386.20 b4, 473.2 b5)	
Light P15	SVGHNGMEVF	23	1076.5	538.75	683.29 b7; 812.34 b8; 911.41 b9	23 (10s); 36 (30s); 37 (80s)
Heavy P15	SVGHNGMEVF*	23	1086.5	543.76	683.29 b7; 812.34 b8; 911.41 b9	
Light P16	NYQIDHHL	14	1039.5	520.25	406.22 y3; 634.33 y5; 762.39 y6 (511.24 b8 2+)	10 (10s); 8 (30s); 14 (80s)
Heavy P16	NYQIDHHL*	14	1046.5	523.76	413.24 y3; 641.35 y5; 769.41 y6 (514.75 b8 2+)	

F*=L-Phenylalanine (¹³C9, ¹⁵N); L*=L-Leucine (¹³C6, ¹⁵N); C(CAM)=Carbamoylmethylated Cysteine; n.a. = not analyzed.

^a Parameters for targeted LC-MS² by PRM: Resolution = 17,500 FWHM at m/z 400; 1.6 Da isolation width; 1 x 10⁵ AGC; HCD CE = 27%; no

^b A mass tolerance of 20 ppm was used to create parallel reaction monitoring chromatograms for peptide detection and integration.

^c Approximate fmol based on normalization of individual peptide peak area responses to the average response of QP peptides.

Table S3. Quantification and Characterization of Intact D6D in Protein Test Samples.

A. Quantification.

	MW Range (Kda)	Quantitative Peptides					Avg fmol	fmol /	µg D6D	D6D as
		QP1	QP2	QP3	QP4	QP5	D6D in	µg	protein	% of
		fmol	fmol	fmol	fmol	fmol	20 µg protein	protein	in 20 µg ^a	Total Protein
<u>D6D batch 101 (20 µg total protein loaded):</u>										
101_ND_Rep1_band1	47-55	222	230	325	96	303	235			
101_ND_Rep1_band2	41-47	569	874	767	680	1010	780			
101_ND_Rep1_band3	31-41	9.9	25	19	23	21	20			
				Sum (bands 1-3)			1035			
101_ND_Rep2_band1	47-55	519	694	489	437	1116	651			
101_ND_Rep2_band2	41-47	361	516	586	539	473	495			
101_ND_Rep2_band3	31-41	9.9	25	19	23	21	20			
				Sum (bands 1-3)			1165			
Replicate Average batch 101:							1100	55	0.057	0.28%
<u>D6D batch 4119 (20 µg total protein loaded):</u>										
4119_ND_Rep1_band1	47-55	966	586	301	517	1156	705			
4119_ND_Rep1_band2	41-47	694	369	119	253	939	475			
4119_ND_Rep1_band3	31-41	37	47	41	17	337	96			
				Sum (bands 1-3)			1276			
4119_ND_Rep2_band1	47-55	871	435	253	416	1033	602			
4119_ND_Rep2_band2	41-47	806	456	138	244	976	524			
4119_ND_Rep2_band3	31-41	36	69	111	8	311	107			
				Sum (bands 1-3)			1232			
Replicate Average batch 4119:							1254	63	0.064	0.32%

^a Based on the MW of delta-6-desaturase of 51,413.

B. Further characterization of intact D6D using non-quantitative peptides.

	MW	non-quantitative peptides						Approx. fmol
	Range	T2	T7	T4	T3	T1	T6	D6D in
	(Kda)	~fmol	~fmol	~fmol	~fmol	~fmol	~fmol	20 µg protein
<i>D6D batch 101 (20 µg total protein loaded):</i>								
101_ND1_bands_1_&_2	41-55	279	174	630	803	1349	920	693
101_ND1_band_3	31-41	124	52	136	121	911	671	336
D6D: Sum of bands 1-3								1028

Table S4. Distribution of D6D Peptides in Gel Bands Following SDS-PAGE of SGF-Digested Test Protein Batch 101.

Sample	MW Range (Kda)	quantitative peptides (QP)					Avg QP1- 5	% Intact D6D ^a	non-quantitative peptides (TP)							
		QP1	QP2	QP3	QP4	QP5			T1	T2	T3	T4	T5	T6	T7	T8
		fmol	fmol	fmol	fmol	fmol			~fmol	~fmol	~fmol	~fmol	~fmol	~fmol	~fmol	~fmol
<u>101 t1 = 3 Sec:</u>																
101_t1a_band1	47-55	33	91	71	20	110	65	14%	51	0	24	29	nd	nd	62	128
101_t1b_band2	41-47	91	142	168	143	168	143	30%	33	0	0	19	nd	nd	83	164
101_t1c_band3	31-41	25	64	78	58	53	56	12%	25	24	nd	8	nd	nd	76	106
<i>D6D: Sum of bands 1-3</i>		149	298	317	222	331	263	55%	110	24	24	57	nd		221	398
101_t1d_band4	21-31	4	18	16	9	0	9	2%	0	0	0	19	nd	nd	188	0
101_t1e_band5	10-21	23	14	23	37	52	30	6%	0	0	370	20	nd	nd	0	0
101_t1f_band6	3-10	12	15	72	46	0	29	6%	103	0	0	0	nd	nd	0	0
<i>Sum All Bands > 3kDa:</i>		189	345	429	314	384	332	69%	213	0	394	96	nd	nd	408	398
101_t1g_band7	1-3	6	0	0	28	0	7	1%	0	0	0	0	nd	nd	0	0
<u>101 t2 = 10 Sec:</u>																
101_t2a_band1	47-55	6	6	7	4	31	11	2%	0	0	0	1	0	0	4	33
101_t2b_band2	41-47	0	20	23	5	8	11	2%	6	0	0	0	0	0	0	0
101_t2c_band3	31-41	0	47	0	10	56	23	5%	0	3	0	0	0	0	0	0
<i>D6D: Sum of bands 1-3</i>		6	72	30	19	96	45	9%	6	3	0	1	0	0	4	33
101_t2d_band4	21-31	27	33	0	21	108	38	8%	0	0	0	16	0	0	0	0
101_t2e_band5	10-21	48	136	237	36	0	91	19%	0	0	0	61	0	297	27	0
101_t2f_band6	3-10	58	0	0	81	0	28	6%	13	0	6	35	0	0	8	0
<i>Sum All Bands > 3kDa:</i>		138	242	267	157	204	202	42%	19	0	6	114	0	420	40	33
101_t2g_band7	1-3	0	0	0	0	0	0	0%	0	0	0	0	0	0	0	0
<u>101 t3 = 30 Sec:</u>																
101_t3a_band1	47-55	0	3	7	4	7	4	1%	0	0	0	0	0	0	0	0
101_t3b_band2	41-47	5	0	11	0	0	3	1%	0	0	0	0	0	0	0	0
101_t3c_band3	31-41	1	0	0	12	0	2	1%	0	0	0	0	0	0	0	0
<i>D6D: Sum of bands 1-3</i>		6	3	18	16	7	10	2%	0	0	0	0	0	0	0	0
101_t3d_band4	21-31	29	87	0	13	9	28	6%	0	0	0	0	0	0	0	0
101_t3e_band5	10-21	56	74	0	60	25	43	9%	10	0	0	63	16	43	6	0
101_t3f_band6	3-10	86	61	248	95	62	110	23%	19	0	1	36	0	201	5	61
<i>Sum All Bands > 3kDa:</i>		176	225	266	184	104	191	40%	29	0	1	99	16	244	11	61
101_t3g_band7	1-3	1	0	0	4	68	0	0%	0	0	0	4	0	0	0	0

^a The % Intact D6D based on average D6D levels from 101 ER t0 control experiments 1 and 2 (480 fmol D6D/8.3 µg total ER protein).

Table S4 cont. Distribution of D6D Peptides in Gel Bands Following SDS-PAGE of SGF-Digested Test Protein Batch 101.

Sample	MW Range (Kda)	quantitative peptides (QP)					Avg QP1-5	% Intact D6D ^a	non-quantitative peptides (TP)								
		QP1	QP2	QP3	QP4	QP5			T1	T2	T3	T4	T5	T6	T7	T8	
		fmol	fmol	fmol	fmol	fmol			~fmol	~fmol	~fmol	~fmol	~fmol	~fmol	~fmol	~fmol	
<u>101 t4 = 80 Sec:</u>																	
101_t4a_band1	47-55	0	0	0	0	0	0	0%	0	0	0	0	0	0	0	0	
101_t4b_band2	41-47	0	0	0	0	0	0	0%	0	0	0	0	0	0	0	0	
101_t4c_band3	31-41	0	0	0	0	0	0	0%	0	0	0	0	0	0	0	0	
D6D: Sum of bands 1-3		0	0	0	0	0	0	0%	0	0	0	0	0	0	0	0	
101_t4d_band4	21-31	0	0	0	23	0	5	1%	0	12	0	0	nd	0	8	0	
101_t4e_band5	10-21	31	20	0	148	0	40	8%	0	0	0	23	nd	0	2	0	
101_t4f_band6	3-10	7	42	0	35	192	55	11%	0	4	0	2	nd	0	10	0	
Sum All Bands > 3kDa:		37	62	0	205	192	99	21%	0	17	0	26	0	0	20	0	
101_t4g_band7	1-3	160	76	180	185	395	199	42%	13	4	0	28	nd	0	12	128	
<u>101 t5 = 5 min:</u>																	
101_1t5a_band1	47-55	0	0	0	0	0	0	0%	0	0	0	0	0	0	0	0	
101_1t5b_band2	41-47	0	0	0	0	0	0	0%	0	0	0	0	0	0	0	0	
101_1t5c_band3	31-41	0	0	0	0	0	0	0%	0	0	0	0	0	0	0	0	
D6D: Sum of bands 1-3		0	0	0	0	0	0	0%	0	0	0	0	0	0	0	0	
101_1t5d_band4	21-31	3	27	0	21	0	10	2%	0	0	0	28	24	0	11	0	
101_1t5e_band5	10-21	1	8	0	2	0	2	0%	0	0	0	9	11	0	0	0	
101_1t5f_band6	3-10	0	0	0	4	0	1	0%	0	0	0	15	3	0	0	0	
Sum All Bands > 3kDa:		4	34	0	27	0	13	3%	0	0	0	52	37	0	11	0	
101_1t5g_band7	1-3	0	0	23	8	0	6	1%	0	0	0	24	14	0	0	0	
<u>101 t6 = 15 min:</u>																	
101_1t6a_band1	47-55	0	0	0	0	0	0	0%	0	0	0	0	0	0	0	0	
101_1t6b_band2	41-47	0	0	0	0	0	0	0%	0	0	0	0	0	0	0	0	
101_1t6c_band3	31-41	0	0	0	0	0	0	0%	0	0	0	0	0	0	0	0	
D6D: Sum of bands 1-3		0	0	0	0	0	0	0%	0	0	0	0	0	0	0	0	
101_1t6d_band4	21-31	0	0	0	0	0	0	0%	0	0	0	6	0	0	0	0	
101_1t6e_band5	10-21	1	8	0	2	0	2	0%	0	0	0	19	nd	0	0	0	
101_1t6f_band6	3-10	2	6	0	4	0	2	1%	0	0	0	6	nd	0	0	0	
Sum All Bands > 3kDa:		3	14	0	6	0	5	1%	0	0	0	31	0	0	0	0	
101_1t6g_band7	1-3	0	10	0	17	0	5	1%	0	0	0	18	nd	0	0	0	

^a The % Intact D6D based on average D6D levels from 101 ER t0 control experiments 1 and 2 (480 fmol D6D/8.3 µg total ER protein).

Table S4 cont. Distribution of D6D Peptides in Gel Bands Following SDS-PAGE of SGF-Digested Test Protein Batch 101.

SGF Sample	MW Range (Kda)	quantitative peptides (QP)							non-quantitative peptides (TP)							
		QP1 fmol	QP2 fmol	QP3 fmol	QP4 fmol	QP5 fmol	Avg of QP1-5	% Intact D6D ^a	T1 ~fmol	T2 ~fmol	T3 ~fmol	T4 ~fmol	T5 ~fmol	T6 ~fmol	T7 ~fmol	T8 ~fmol
<u>101 t7 = 60 min:</u>																
101_1t7a_band1	47-55	0	0	0	0	0	0	0%	0	0	0	0	0	0	0	0
101_1t7b_band2	41-47	0	0	0	0	0	0	0%	0	0	0	0	0	0	0	0
101_1t7c_band3	31-41	0	0	0	0	0	0	0%	0	0	0	0	0	0	0	0
D6D: Sum of bands 1-3		0	0	0	0	0	0	0%	0	0	0	0	0	0	0	0
101_1t7d_band4	21-31	0	0	0	0	0	0	0%	0	0	0	0	0	0	0	0
101_1t7e_band5	10-21	0	0	0	0	0	0	0%	0	0	0	0	0	0	0	0
101_1t7f_band6	3-10	0	0	0	0	0	0	0%	0	0	0	0	0	0	0	0
Sum All Bands > 3kDa:		0	0	0	0	0	0	0%	0	0	0	0	0	0	0	0
101_1t7g_band7	1-3	0	10	0	9	0	4	1%	0	0	0	8	1	0	0	11

^aThe % Intact D6D based on average D6D levels from 101 ER t0 control experiments 1 and 2 (480 fmol D6D/8.3 µg total ER protein).

Table S5. Distribution of Additional D6D Peptides in Protein Gel Bands After SGF Digestion.

Detection of peptides formed from cleavage by pepsin in SGF followed by in-gel cleavage by trypsin.

	peptides:	PT2	PT3	PT4	PT5	PT7	PT8	PT10	PT11	PT12	PT13	PT15	PT16	PT18
		~fmol	~fmol	~fmol	~fmol	~fmol	~fmol	~fmol	~fmol	~fmol	~fmol	~fmol	~fmol	~fmol
	MW													
	Range													
	(kDa)													
<u>Non-digested 101:</u>														
101_ND_bands1&2	41-55	0	0	0	0	0	0	0	0	0	0	0	0	0
101_ND_band3	31-41	0	0	0	0	0	0	0	0	0	0	0	0	0
101_ND_band4	21-31	0	0	0	0	0	0	0	0	0	0	0	0	0
<u>Non-digested 4119:</u>														
4119_ND_band5	10-21	0	0	0	0	0	0	0	0	0	0	0	0	0
4119_ND_band6	3-10	0	0	0	0	0	0	0	0	0	0	0	0	0
4119_ND_band7	1-3	0	0	0	0	0	0	0	0	0	0	0	0	0
<u>101 t1 = 3-5 Sec:</u>														
101_t1a+b_bands1&2	41-55	0	0	0	0	15	0	0	0	0	0	0	0	0
101_t1c_band3	31-41	2	0	0	0	0	13	0	0	0	0	0	0	0
101_t1d_band4	21-31	0	0	0	0	0	0	0	0	0	0	0	0	0
101_t1e_band5	10-21	0	0	0	0	0	0	0	0	0	0	0	0	0
101_t1f_band6	3-10	0	0	0	0	0	0	0	0	0	0	0	0	0
101_t1g_band7	1-3	0	0	0	0	0	0	0	0	0	0	0	0	0
<u>101 t2 = 10 Sec:</u>														
101_t2a+b_bands1&2	41-55	0	0	0	0	0	0	0	0	0	0	0	0	0
101_t2c_band3	31-41	0	0	0	0	0	12	0	0	0	0	0	0	0
101_t2d_band4	21-31	0	0	0	0	0	7	0	0	0	0	0	0	0
101_t2e_band5	10-21	12	0	0	0	24	11	0	0	0	0	0	0	0
101_t2f_band6	3-10	0	0	0	0	0	7	0	0	0	0	0	0	0
101_t2g_band7	1-3	0	0	0	0	0	0	0	0	0	0	0	0	0

Table S5 cont. Distribution of Additional D6D Peptides in Protein Gel Bands After SGF Digestion.

Detection of peptides formed from cleavage by pepsin in SGF followed by in-gel cleavage by trypsin.

	peptides:	PT2	PT3	PT4	PT5	PT7	PT8	PT10	PT11	PT12	PT13	PT15	PT16	PT18
		~fmol	~fmol	~fmol	~fmol	~fmol	~fmol	~fmol	~fmol	~fmol	~fmol	~fmol	~fmol	~fmol
	MW													
	Range													
	(kDa)													
<u>101 t3 = 30 Sec:</u>														
101_t3a+b_bands1&2	41-55	0	0	0	0	0	0	0	0	0	0	0	0	0
101_t3c_band3	31-41	0	0	6	0	0	0	4	0	0	0	0	0	0
101_t3d_band4	21-31	0	0	0	0	3	0	16	0	0	0	0	0	0
101_t3e_band5	10-21	0	60	0	10	13	0	0	0	0	0	0	0	0
101_t3f_band6	3-10	0	107	0	0	0	2	0	0	0	0	0	0	0
101_t3g_band7	1-3	0	0	0	0	0	0	0	0	0	0	0	0	0
<u>101 t4 = 80 Sec:</u>														
101_t4a+b_bands1&2	41-55	0	0	0	0	0	0	0	0	0	0	0	0	0
101_t4c_band3	31-41	0	0	0	0	0	6	0	0	0	0	0	0	0
101_t4d_band4	21-31	0	0	0	0	22	3	0	0	0	0	0	0	0
101_t4e_band5	10-21	0	0	0	0	17	3	0	0	0	0	0	0	0
101_t4f_band6	3-10	2	0	0	0	0	0	14	0	0	0	0	0	1
101_t4g_band7	1-3	0	0	0	0	0	0	0	0	0	0	0	0	0
<u>101 t5 = 5 min:</u>														
101_t5a+b_bands1&2	41-55	0	0	0	0	0	0	0	0	0	0	0	0	0
101_t5c_band3	31-41	0	0	0	0	0	0	0	0	0	0	0	0	0
101_t5d_band4	21-31	0	0	0	0	0	0	6	0	0	0	0	0	0
101_t5e_band5	10-21	0	0	0	0	16	0	2	0	0	0	0	0	0
101_t5f_band6	3-10	0	0	0	0	0	0	15	0	0	0	0	0	0
101_t5g_band7	1-3	0	0	0	0	0	0	0	0	0	0	0	0	0

Table S5 cont. Distribution of Additional D6D Peptides in Protein Gel Bands After SGF Digestion.

Detection of peptides formed from cleavage by pepsin in SGF followed by in-gel cleavage by trypsin.

	peptides:	PT2	PT3	PT4	PT5	PT7	PT8	PT10	PT11	PT12	PT13	PT15	PT16	PT18
		~fmol	~fmol	~fmol	~fmol	~fmol	~fmol	~fmol	~fmol	~fmol	~fmol	~fmol	~fmol	~fmol
	MW													
	Range													
	(kDa)													
<u>101 t6 = 15 min:</u>														
101_t6a+b_bands1&2	41-55	0	0	0	0	0	0	0	0	0	0	0	0	0
101_t6c_band3	31-41	0	0	0	0	0	0	0	0	0	0	0	0	0
101_t6d_band4	21-31	0	0	0	0	0	0	0	0	0	0	0	0	0
101_t6e_band5	10-21	7	0	0	0	0	0	3	0	0	0	0	0	0
101_t6f_band6	3-10	0	0	0	0	0	0	10	0	0	48	0	0	0
101_t6g_band7	1-3	0	0	0	0	9	0	0	0	0	0	0	6	0
<u>101 t7 = 60 min:</u>														
101_t7a+b_bands1&2	41-55	0	0	0	0	0	0	0	0	0	0	0	0	0
101_t7c_band3	31-41	0	0	0	0	0	0	0	0	0	0	0	0	0
101_t7d_band4	21-31	0	0	0	0	0	0	0	0	0	0	0	0	0
101_t7e_band5	10-21	0	0	0	0	0	0	0	0	0	0	0	0	0
101_t7f_band6	3-10	0	0	0	0	0	0	0	0	0	0	0	0	0
101_t7g_band7	1-3	0	0	0	0	0	0	0	0	0	0	0	0	4

Figure S1. Copy of earlier work presented at 2010 ASMS annual conference.

Alvarado, R.; Dicely, I.; Mai, K.; Goodstal, J.; Facciotti, D.; Phinney, B. A targeted proteomics approach to quantitate the levels of delta-6 desaturase in developing safflower seeds. 58th Annual ASMS conference on mass spectrometry and allied topics, 2010, May 23-27, Salt Lake City, UT, USA.



A Targeted Proteomics Approach to Quantitate the Levels of Delta 6-desaturase in Developing Safflower Seeds

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INTRODUCTION

Delta 6-desaturase, a transmembrane protein, catalyzes the synthesis of gamma-linolenic acid (GLA) from linoleic acid. GLA offers dietary support for a wide range of conditions such as cardiovascular health, Osteoporosis, Rheumatoid Arthritis, Eczema and neurological disorders. Delta-6-desaturase converts linoleic acid into GLA, a fatty acid otherwise absent from safflower oil. A gene from *Sapropignia diclina* (*S. diclina*) coding for a delta-6 desaturase was engineered into safflower for the production and commercialization of high GLA safflower oil, now commercialized as SONOVA™ 400. To date, attempts to raise antibodies against this delta-6-desaturase have failed; therefore a targeted proteomics approach was necessary to quantitate the expressed protein. We used this approach to measure its levels in the microsomal fractions of developing and maturing transgenic GLA safflower seeds (7 days, 18 days, 21 days, 28 days, 35 days, and 42 days post anthesis [DPA]).

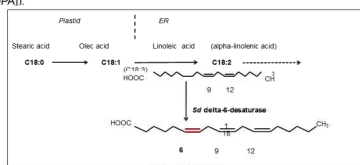


Figure 1. Shows the pathway for the conversion of Gamma-linolenic acid from Linoleic acid. The reaction is catalyzed by a transmembrane protein called Delta-6-desaturase.

METHODS

Protein Extraction Methods

Microsomes were prepared as described in the scientific literature (Taton 1996). Briefly, 12g of frozen seeds were crushed in a chilled mortar in the presence of extraction buffer (50mM Tris-HCl, 10% glycerol, 35mM potassium sulfate, 250mM mannitol, 2mM EGTA, 1mM aminocaproic acid, 1mM benzimidazole HCl, 2.8% PVPP, 10% ascorbic acid, 2mM DTT, and protease inhibitor at 1/1,000 volume, pH 7.3). The buffer was added gradually during grinding for a final 10:1 ratio of buffer/seed (v/w). Sand (3 - 4g) was added as an abrasive to facilitate the grinding of the tissue. The resulting crude suspension was filtered through 4 layers of cheesecloth and centrifuged at 3,000 x g, then 12,000 x g to remove plant debris. Microsomes were separated from soluble enzymes by centrifugation at 106,000 x g. The microsome pellet was resuspended in resuspension buffer (0.25M mannitol, 10% glycerol, 5mM Tris-HCl pH 7.2, and 1mM DTT). In the MudPIT experiment, it was further fractionated by centrifugation over a discontinuous sucrose gradient (20%, 34%, and 40% sucrose in resuspension buffer). Samples were collected at each step to evaluate protein content by Bradford assay for total protein preparations or by BCA assay for microsomal fractions. Recovered endoplasmic reticulum (ER) microsomes equaled approximately 2.7% of the total protein from seeds sampled at 20 DPA. Protein concentrations were confirmed using SDS-PAGE. The ER-enriched fraction (ER fraction) at the interface between 20% and 34% sucrose was collected and used for both identifying delta-6-desaturase by MudPIT and Targeted Proteomics. In SRM experiments performed with crude oil, mature seeds or seeds from the time course experiment, the microsome preparations were floated over a 40% sucrose resuspension buffer. The microsome preparation was then resuspended in resuspension buffer and centrifuged at 106,000 x g for 30 minutes. The pellet was then resuspended in 20% ACN prior to tryptic digestion.

Digestion Methods

50µg of total protein (quantitation was performed by Amino Acid Analysis) was resuspended in 50µL of 20% ACN. 1.5µg of unmodified Trypsin was added and the sample was digested in a scientific microwave (CEM, Mathews, NC). During the entire digestion method, the temperature is monitored by a fiber optic probe and held constant at 50 degrees Celsius. During the first cycle, the microwave power is increased to 50 watts for 2 minutes and 30 seconds. During the second cycle, the microwave power is decreased to 1 watt for 10 minutes. During the third and final cycle, the temperature is decreased to room temperature for 20 minutes. The sample was then dried down in a vacuum centrifuge. The sample was resuspended in 50µL of 2% ACN/0.1% TFA, sonicated for 10 minutes, and then centrifuged for 8 minutes at 13,200 rpm. 10µL of the sample was then used for LC/MS analysis.

LC/MS Methods

The Paradigm MG4 II IPLC System (Michrom Bio Resources, Auburn, CA) coupled with a Thermo TSQ Vantage triple quadrupole mass spectrometer (Thermo, San Jose, CA) through a Michrom Captive spray ionization source was used for peptide separation and analysis. 10µg of each digested sample was loaded onto a trap column (Zorbax 300SB-C18, 5µm, 5 mm x 0.3 mm; Agilent, Santa Clara, CA) and desalted. Peptides were then eluted from the trap, separated with a reverse phase Michrom Magic C18AQ (0.2 x 150 mm) capillary column at a flow rate of 2µL per min. Peptides were eluted using a 45 min gradient of 2% B to 35% B over 23 minutes, 35% B to 80% B for 11 minutes, held at 80% B for 1 minute, 80% B to 5% B in 1 minute, and re-equilibrated for 9 minutes at 5% B (A = 0.1% Formic Acid, B = 100% Acetonitrile) and directly sprayed into the mass spectrometer. The mass spectrometer was operated with a spray voltage 1.8kV, a heated capillary temperature of 180°C, and a full scan range of 350-1400 m/z. Q1 and Q3 were both set to unit resolution (0.7 Da FWHM). Collision Energy (CE) and S-lens values were specifically optimized for each transition.

The peptides that we chose to target were derived from previous MudPIT experiments. We chose two peptides to target: SQSDFIASYR and LQVLSTR. Both peptides were isotopically labeled and optimized by directly injecting the peptide standards into the mass spectrometer. The isotopically labeled peptides were spiked into each sample that was analyzed, which allowed us to quantitate the relative levels of delta-6-desaturase in each sample.

#	Symbol	Accession	Protein	Score	Rank
1	Symbol: None 1 ATP synthase beta chain 1, mito...	A15G08670...	60 kDa	18	17
2	Symbol: None 1 Lamin binding protein 1 (BOP-1...	A15G08543...	71 kDa	19	18
3	Symbol: None 1 ATP synthase alpha chain, mito...	A15G07698...	86 kDa	11	9
4	Symbol: None 1 ADP, ATP carrier protein 1, mito...	A15G08580...	41 kDa	10	14
5	Symbol: None 1 succinate dehydrogenase (ubiq...	A15G04765...	70 kDa	16	13
6	Symbol: None 1 ATPase, plasma membrane-ty...	A15G07950...	106 kDa	10	8
7	Symbol: None 1 heat shock protein 70, popul...	A15G12580...	71 kDa	11	11
8	Symbol: EM2207 1 60S ribosomal protein L3 (R...	A15G14317...	45 kDa	5	4
9	Symbol: None 1 actin 1 (ACT1), identical to PP...	A15G07626...	42 kDa	12	10
10	Symbol: None 1 shepherd protein (SHP)	A15G04190...	94 kDa	6	4

Figure 2. In prior MudPIT experiments, *S. diclina* delta-6-desaturase was identified in transgenic microsomal samples from GLA safflower seeds at 20 DPA, but not in the non-transgenic Centennial-derived microsomes.

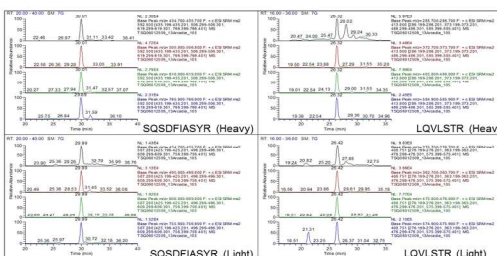


Figure 3. Chromatograms showing both *S. diclina* delta-6-desaturase peptides that were targeted as well as their corresponding isotopically labeled counterparts.

CONCLUSIONS

Due to the lack of commercially available antibodies for *S. diclina* delta-6-desaturase, an immunoassay could not be employed. Therefore, the development of a targeted proteomics approach to identify and quantitate this transgenic enzyme was necessary. Using this approach, we have shown that *S. diclina* delta-6-desaturase is present exclusively in microsomes (ER) from safflower seeds. After rapid increase during seed development the enzyme then gradually decreases as the seed matures and desiccates until it is no longer detected in mature, dry seeds nor, consequently, in SONOVA™ 400 protein extracts (Fig. 4). These results paralleled the gene expression levels of the *S. diclina* delta-6-desaturase across seed development, maturation, and desiccation (Fig. 5). This approach was very direct and very efficient in generating results with the desired degree of sensitivity, accuracy, and precision. Most of the results presented in this poster were also presented in a successful premarket notification for a New Dietary Ingredient, approved by the FDA in 2009. To our knowledge, this was the first time a targeted proteomics approach was used in a successful petition to the FDA.

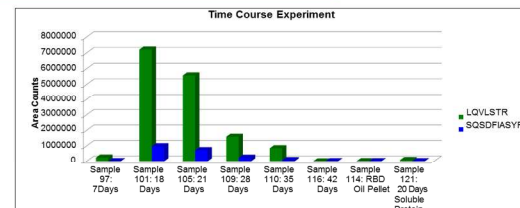


Figure S2. SDS-PAGE of SGF-digested control ER and transgenic ER batch 101. (A) Non-transgenic control ER (15 μ g total protein/lane): lane M, protein MW markers; lane 1, non-digested control ER; lanes 2-8, control ER after digestion in SGF for 0 sec, 10 sec, 30 sec, 80 sec, 5 min, 15 min, 30 min, and 60 min. (B) Transgenic ER batch 101 (8.3 μ g total protein/lane): lane M, protein MW markers; lanes 1 and 2, non-digested ER 101; lane 3, blank; lanes 4-10, ER 101 after digestion in SGF for 3 sec, 10 sec, 30 sec, 80 sec, 5 min, 15 min, and 60 min.

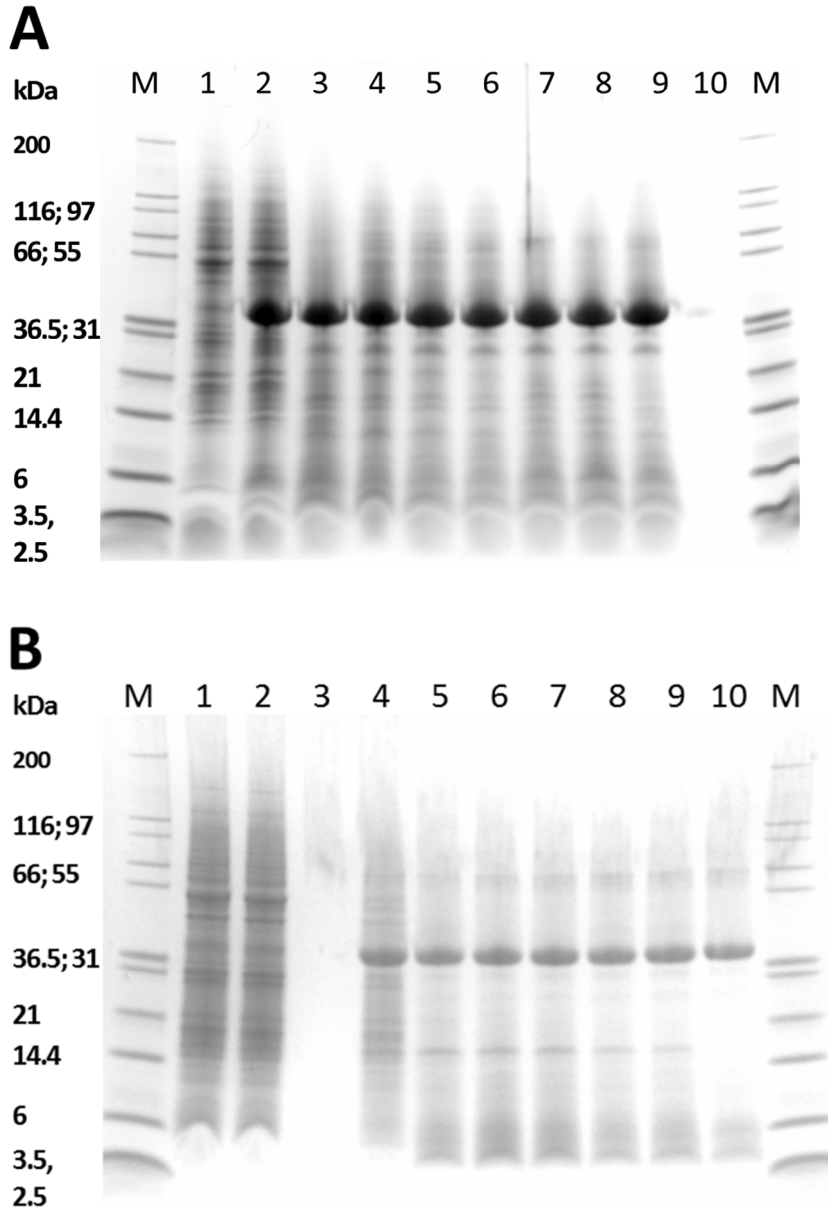
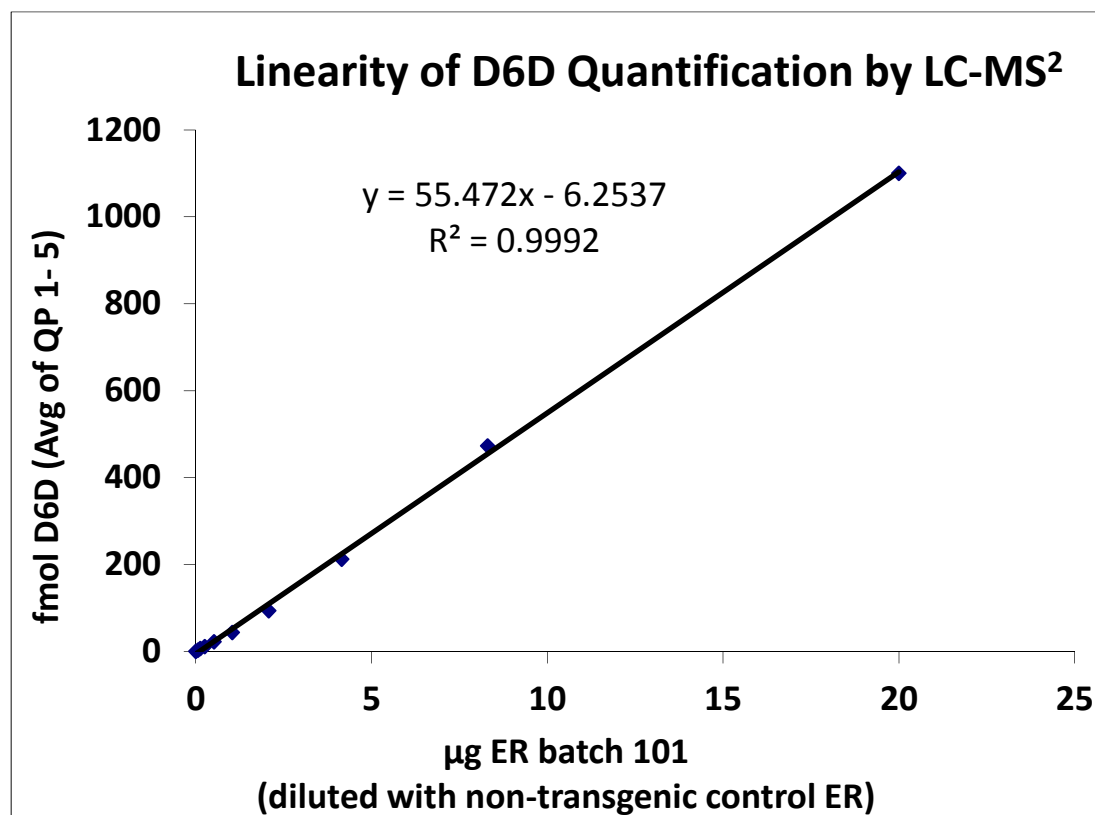


Figure S3. Linearity of D6D quantification by LC-MS² from analysis of ER batch 101 diluted in non-transgenic control ER.



Linearity of D6D LC-MS2 Quantification Method

Calibrator	µg ER 101 protein	% of original 20 µg ER 101 quantification	nominal fmol	fmol measured (Avg of QP 1-5)	% of Nominal fmol
D6D_Cal00 ^a	20	100	1100	1100	N.A. ^a
D6D_Cal01 ^b	8.3	41.5	457	472	104%
D6D_Cal02	4.15	20.75	228	212	93%
D6D_Cal03	2.075	10.375	114	94	82%
D6D_Cal04	1.0375	5.1875	57	44	77%
D6D_Cal05	0.51875	2.59375	29	22	77%
D6D_Cal06	0.259375	1.296875	14	11	79%
D6D_Cal07	0.1296875	0.6484375	7	6.6	92%
D6D_Cal08	0.06484375	0.32421875	4	2.8	79%
WT_Blank	0	0	0	0.0	N.A.

^a These data for 20 µg Cal00 are from original quantification and characterization of D6D from 20 µg/lane SDS-PAGE of ER batch 101, D6D gel bands (replicate average).

^b Cal01 (8.3 µg) represents the amount of SGF-digested ER batch 101 loaded per gel lane, for evaluation of D6D digestibility.

Figure S4. Additional peptic peptides identified by direct LC-MS² using Q-Exactive MS.

A. Peptide P8: VGVMVGNL

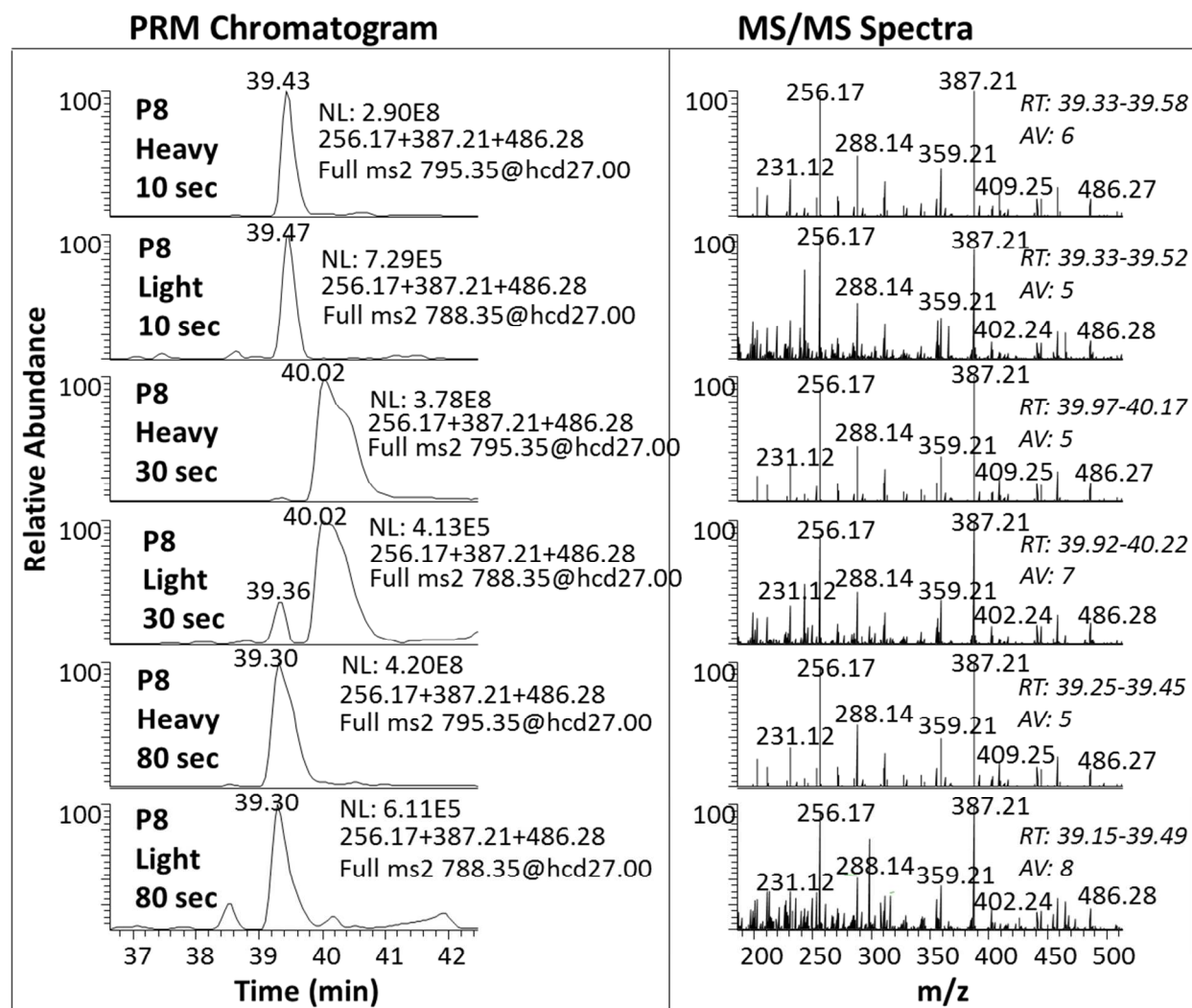


Figure S4 cont. Additional peptic peptides identified by direct LC-MS² using Q-Exactive MS.

B. Peptide P10: HGDPDIDTMPIL

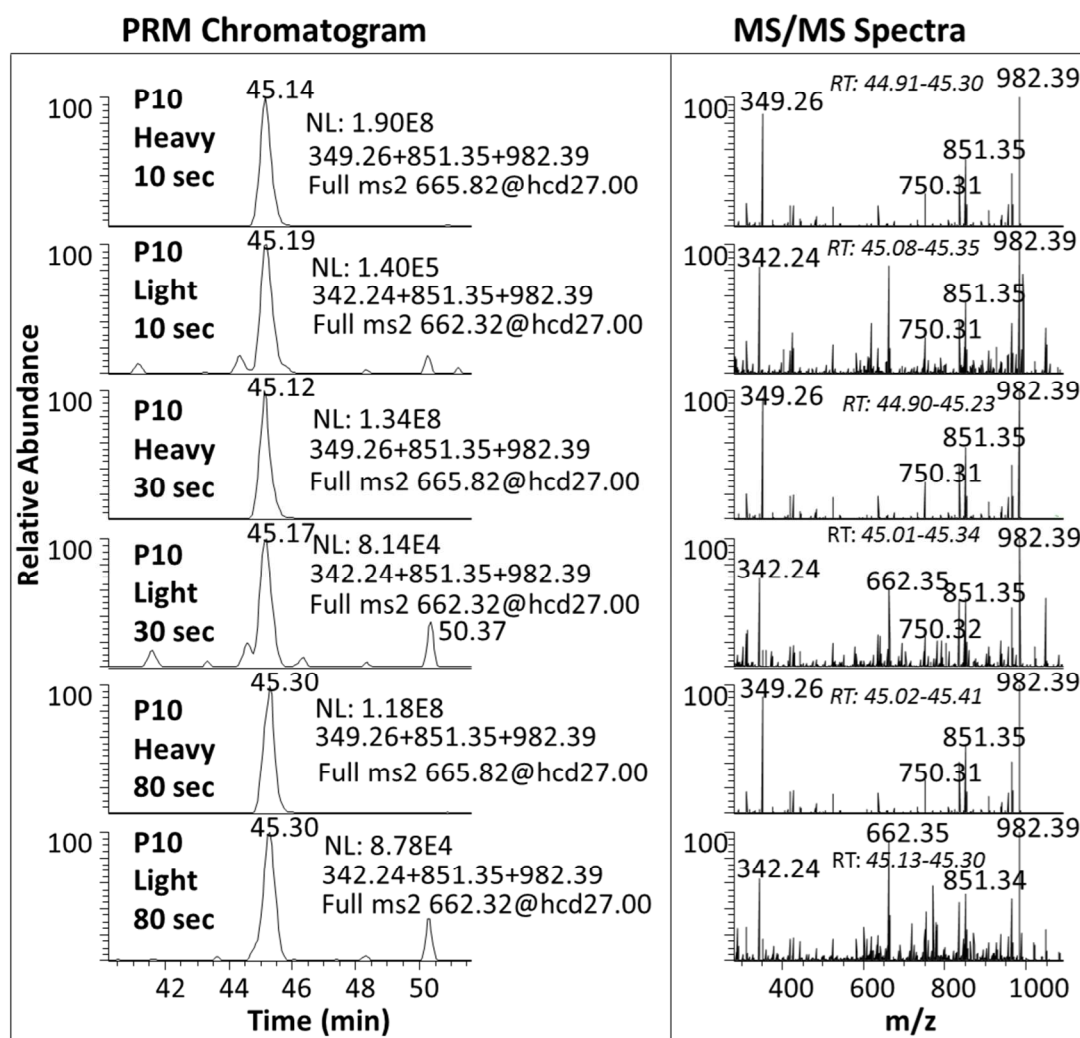


Figure S4 cont. Additional peptic peptides identified by direct LC-MS² using Q-Exactive MS.

C. Peptide P12: VYAANMSL

