Appendix X

In vitro Pepsin Digestibility Study

STUDY TITLE

Soy Leghemoglobin Preparation: *in vitro* digestibility study in human simulated gastric fluid (pH 2.0) at two different pepsin-protein ratios, 10 units per μg and 1 unit per μg

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LABORATORY STUDY ID

REG 2016-Pepsin LegHb

Statement of No Data Confidentiality

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Company

Company Agent:

Impossible Foods, Inc.

10/13/16
Date

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Good Laboratory Practice Compliance Statement

This study was not conducted and reported in compliance with the requirements of the Good Laboratory Practice Standards (40 CFR Part 160) of the Code of Federal Regulations of the United States of America. This is a characterization assessment of the similarity of the introduced proteins to known and putative allergens based on source of the genes and the sequences of the proteins. There is no test system. However, raw data including PubMed searches and bioinformatics comparisons were archived in PDF format in the Authors laboratory with a copy given to the sponsor.

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SUMMARY

The Soy Leghemoglobin (LegHb) protein used in this study was produced in *Pichia pastoris* and supplied by Impossible Foods, Inc. of Redwood City, CA. The gene was originally derived from soybean (*Glycine max*) and encodes the 145 amino acid protein sequence listed as Accession number P02236 in the UniProt protein database. The Soy Leghemoglobin Preparation test material was supplied as a liquid protein solution by the study sponsor (Impossible Foods, Inc.) with assurance of the soy leghemoglobin protein identity and purity being approximately 66%, with the remaining 34% of proteins from the host, *Pichia pastoris* (synonym *Komagataella pastoris*).

The Soy Leghemoglobin Preparation was supplied as a concentrated aqueous solution and was subjected to digestion in pepsin based on the protocol in Thomas *et al.* (2004), as refined by Ofori-Anti et al., 2008 with minor modifications. The time to reach 90% digestion of the protein by pepsin was estimated as the first sample time having less than 10% residual protein primary protein compared to diluted non-digested sample protein. The ability of the assay to detect 10% residual protein was determined prior to the digestion tests using serial dilutions of the test protein in a similar SDS-PAGE, Coomassie blue staining to ensure that a residual of 10% undigested control sample detectable under the conditions used for the study. The primary LegHb band migrated at ~ 13 kDa in SDS-PAGE. Pepsin was diluted in simulated gastric fluid (SGF) with the pH adjusted to 2.0. The pepsin solution was tested for proteolytic activity by digestion of hemoglobin within 24 hours of each assay day. The mass ratio of pepsin to LegHb preparation was adjusted to achieve ~ 10 units of pepsin activity per microgram of total protein in solution. An additional assay was performed with the ratio of 1 unit of pepsin activity per microgram of test proteins. Digestions were performed at 37°C under timed conditions. Samples of the digestion mixtures were removed and neutralized at various time points from 30 seconds to 60 minutes and samples of each were electrophoresed in SDS-PAGE and stained with Coomassie blue to evaluate digestion completeness.

The results of this study demonstrated that the *P. pastoris*-produced LegHb protein and the *Pichia* host proteins within the Soy Leghemoglobin Preparation were rapidly digested in pepsin at pH 2.0 at both ratio of 1 µg in 10 units (as per standard protocol) and 1 µg in 1 unit pepsin activity (as an experimental protocol). The SDS-PAGE Coomassie blue gel staining method demonstrated that more than 90% of the *P. pastoris*-produced LegHb protein and the *Pichia* host proteins were digested in less than 2 minutes in replicate assays. No degradation bands were found to result from digestion of the LegHb protein or the *Pichia* proteins. Therefore, our conclusion is that the *P. pastoris* produced LegHb and the *Pichia* host proteins are rapidly digested at both ratio of 1 µg in 10 units and 1 µg in 1 unit activity of pepsin at pH 2 and that no pepsin-stable fragments were identified in the assay. Based on Codex (2003) guidelines for the allergenicity assessment, there is no added concern of risk based on stability of this LegHb and *Pichia* protein preparation in pepsin.

Table of Contents

Section **Page**

Figures

Abbreviations

aa	amino acid
$A_{280\ nm}$	Absorbance of light at a wavelength of 280 nm
BSA	Bovine serum albumin
CFR	Code of Federal Regulations
D0-60	Digestion samples (hemoglobin plus pepsin) from time 0 (quenched prior to digestion) to time 60 min
E0	Experimental control pepsin without the hemoglobin, time 0
E60	Experimental control pepsin without the hemoglobin, 60 min
Hb	Bovine blood hemoglobin
kDa	kilodalton
LegHb	Leghemoglobin from *Glycine max* (Soy), *Pichia pastoris* produced
LOD	Lower limit of detection
ma	milliampere
mg	milligram
ml	milliliter
mM	millimolar
µl	microliter
na	Not applicable
ng	nanogram
OVA	Ovalbumin
P0	Experimental control protein without pepsin, time 0
P60	Experimental control protein without pepsin, 60 min
PAGE	Polyacrylamide gel electrophoresis
P1/10	Experimental control protein at 10% loading
R^2	Square of the correlation coefficient
SDS	Sodium dodecyl sulfate
SGF	Simulated gastric fluid (without pepsin)
SOP	Standard operating procedure
T	Time point
TCA	Trichloroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
v/v	solute volume to solution volume
w/v	solute weight to solution volume

1. Introduction

Impossible Foods, Inc. of Redwood City, CA is developing a potential food product that contains a hemoglobin protein from soybean (*Glycine max*), called Soy Leghemoglobin (LegHb), as the primary protein ingredient. Impossible Foods, Inc. sponsored tests and an evaluation of the potential allergenicity of the Soy Leghemoglobin preparation, which contains Soy Leghemoglobin protein and proteins from the *Pichia pastoris* production host, in order to consider whether there is a risk of food allergy associated with consumption of the proteins. This report describes the rationale, test methods for testing the protein and results from an *in vitro* digestion assay intended to provide data relative to potential risks of food safety.

The *Codex Alimentarius* Commission guidelines for assessing the allergenicity of GM plants (2003) recommends assessing the introduced protein for stability in pepsin at acidic pH using standard conditions as an assay to help evaluate whether the introduced protein is likely to either increase the rate of sensitization to the host crop, or increase the likelihood of eliciting an allergic response in food allergic consumers. The pepsin stability assay is one study in a weight of evidence approach intended to assess the potential allergenicity of genetically modified crops (Codex, 2003). The test method for the assessment was first described by Astwood *et al.* (1996). The assay is not meant to predict whether a given protein will always be digested in the stomach of the human consumer, but the assay does provide a simple *in vitro* correlation to evaluate protein digestibility. Investigation of proteins that have been tested suggest a marked positive predictive value that food allergens causing systemic reactions are relatively stable in the assay, while non-allergenic food proteins are typically digested relatively quickly (Bannon *et al.*, 2002). Purified porcine pepsin has been used to evaluate the stability of a number of food allergens and non-allergenic proteins in a multi-laboratory study that demonstrated the rigor and reproducibility in nine laboratories (Thomas *et al.*, 2004). Porcine pepsin is an aspartic endopeptidase with broad substrate specificity. Pepsin is optimally active between pH 1.2 and 2.0, but markedly less active at pH 3.5 and irreversibly denatured at pH 7.0 (Collins and Fine, 1981; Crevieu-Gabriel *et al.*, 1999). The assay is performed under standard conditions of 10 units of pepsin activity per microgram of test protein. An additional assay was performed using 1 unit of activity per microgram, which is one-tenth the published standard activity ratio. A relatively pure form of pepsin was used for this assay from Worthington Biochemical Co., pepsin A, product LS003319.

The original assay described by Astwood *et al.* (1996) recommended performing the digestion at pH 1.2, however, the FAO/WHO (2001) suggested using two pH conditions (pH 1.2 and pH 2.0). In comparing pH 2.0 vs. pH 1.2, Thomas *et al.* (2004) showed that protein digestion at pH 2.0 resulted in slightly slower rates of full-length protein and fragment degradation, but did not alter the overall sensitivity of a protein to digestion. Results at pH 1.2 were more consistent than at pH 2.0, with 91% and 77% agreement between laboratories, respectively. However, more recently, we have digested a number of proteins at both pH 1.2 and 2.0 and have did not

demonstrate significant differences (Ofori –Anti et al., 2008). Therefore in this study we only evaluated stability of the protein at pH 2.0.

The digestion was performed at 37°C and samples are removed at specific times and the activity of pepsin is quenched by neutralization with carbonate buffer and Laemmli loading buffer, then heating to more than 85°C for 10 minutes. The timed digestion samples are separated by SDS-PAGE and stained with Coomassie blue to evaluate the extent of digestion. A review of the digestibility assay by Bannon *et al.* (2002) and by Thomas *et al.* (2004) indicates that most of the non-allergenic food proteins that have been tested are digested in around 30 seconds, while many major food allergens are stable, or produce pepsin-stable fragments that are visible for eight to 60 minutes in this assay.

Assay parameters used in this study included verification of pepsin activity, established limit of detection of the protein in the stained gel (at 10% total stainable protein) and use of an objective measurement of the time of digestion required to reach 90% digestion as described by Ofori-Anti et al. (2008). The activity of the pepsin in SGF was tested on each day of assay based on digestion of bovine hemoglobin, as described by Worthington, to ensure that it is within a tolerance interval reported by Worthington for that lot of enzyme. The results of our activity assay did not exactly duplicate the labeled activity determined by Worthington for the lot, but did fall within the acceptance criterion of the Worthington certified activity, plus or minus 1,000 activity units per mg of pepsin. A second important criterion included in our standard operating procedure (SOP) is an objective measured level of residual test protein (LegHb in this case) that must be reached in determining the time of digestion. We defined the time of digestion required to achieve 90% reduction in stained band intensity as the time-point when the residual is less than or equal to 10% of the amount of test protein in the initial sample. To accomplish that a dilution series of test protein is tested in the same SDS-PAGE and colloidal blue staining system as the digests are analyzed with to evaluate a limit of detection (LOD). The LOD must be lower than 10% to perform this assay. The analytical gel for the pepsin digests includes a 10% test protein sample mixed with quenched pepsin (high pH, to avoid digestion). Details and results of the study are reported here.

2. Materials

2.1 Test Substance

The test substance for this study was Soy Leghemoglobin Preparation, which contains leghemoglobin from soybean (*Glycine max)* expressed recombinantly in *Pichia pastoris* (strain MXY0291). The sample was provided by Impossible Foods' from the production run PP-PGM2-15-320-101. The protein sample was in solution in a 50 ml screw cap disposable polypropylene centrifuge tube, shipped on ice packs. The total protein was labeled as 79 mg/ml and the Soy Leghemoglobin composed 66% of total protein calculated by HPLC, according to the certificate of analysis. The buffer indicated by Impossible Foods was 200 mM NaCl. The concentration was evaluated in our lab

using GE 2D Quant assay and determined to be 79.94 mg/ml. Although the predicted molecular weight of LegHb is 15.5 kDa, LegHb migrates at ~13 kDa on SDS-PAGE. Impossible Foods has measured the intact mass of LegHb using mass spectrometry and confirmed that *Pichia pastoris* expresses the full-length form of the protein excluding the N-terminal methionine. Exclusion of the N-terminal methionine is common in microbial protein expression and does not affect protein function. The solution was aliquoted and stored at -20 °C.

2.2 Control Substance

The control substances for this study were bovine hemoglobin, bovine serum albumin (BSA) and chicken ovalbumin (OVA). Each was tested in separate digestion assays to demonstrate the validity based on previous tests and results. The control substance tests were performed prior to the testing the samples.

2.3 Reference Substance

There was no reference substance for this study. Analytical reference standards (e.g., molecular weight markers) used in this study were documented in the data and are described in this report.

2.4 Characterization of Test, Control, and Reference Substances

Characterization of the Soy Leghemoglobin Preparation PP-PGM2-15-320-101 was the responsibility of Impossible Foods, Inc. Impossible Foods shared the molecular weight and the protein (amino acid sequence) with us prior to the study, which was important in analyzing results.

2.5 Critical Analytical Reagents

- Pepsin A, Worthington Biochemical Corporation, product #3319, lot #35B15585, certified as having 2,810 activity units per mg solid
- SGF without pepsin: A 35 mM NaCl solution is adjusted in pH to 2.0 as measured with a calibrated pH meter, using 6.1 N HCl.
- SGF plus pepsin 4000 U: Dissolved the mass of powdered pepsin in SGF to achieve a final activity of 4,000 units per 1.52 mL of SGF, based on the activity units from Worthington, which is 10 units activity per 1 µg of tested protein.
- SGF plus pepsin 400 U: Dissolved the mass of powdered pepsin in SGF to achieve a final activity of 400 units per 1.52 mL of SGF, based on the activity units from Worthington, which is 1 unit activity per 1 µg of tested protein.
- Bovine Serum Albumin (BSA) from Sigma Chemical Co., product #A9647-100G, lot #SLBP1123V.
- Ovalbumin (OVA), from Worthington Biochemical Corporation, product #3054, lot #52P13864.

- Hemoglobin (Hb) from bovine blood, Sigma Chemical Co., product #H2625 -25 G, lot #SLBD9300V is used to test protein pepsin activity and pepsin digestibility.
- Limit of detection determination diluent: Mixed 40 ml of SGF, pH 2.0 with 14.7 ml of carbonate buffer, pH 11.0. $NaHCO_3$, Fisher Scientific, cat #S78284, lot #AD-10033-32.
- Pepsin quenching solution: 200 mM $NaHCO_3$, pH 11
- 6X Laemmli buffer, Boston BioProducts, CAS #BP-111NB, lot #J20Z4R.
- β-mercaptoethanol, BioRad #161-0710, lot #210009868
- Precision Plus Protein™ Dual Xtra Standards from BIO-RAD, product #161-0377, control #64046347
- Novex 10-20% tris-glycine polyacrylamide gels, 1.5 mm thick, 15 wells (Invitrogen EC61385BOX), lot #16022941.
- Tris-Glycine-SDS 10 x running buffer, cat #BP1341-4L, lot #153375.
- BIO-RAD Coomassie Brilliant Blue R-250 staining solution, cat #161-0436, control #200005684.
- BIO-RAD Coomassie Brilliant Blue R-250 destaining solution, cat #161-0438, control #210012192.

3. Test System.

The test system for this study was an *in vitro* digestion model using pepsin in simulated gastric fluid (SGF). Standard Operating Procedures (SOPs) for preparation of the SGF, determination of the detection limit assay, pepsin activity assay, digestion assay, SDS-PAGE and gel staining are on record in the laboratory. The SGF preparation and digestion procedures were based on the methods described by Thomas *et al.* (2004) as modified by Ofori-Anti et al., (2008).

The pepsin activity assay was based on the method described by Worthington for determining the activity of pepsin. An appropriate mass of pepsin powder was dissolved in prepared SGF, pH 2.0 to provide 0.9 mg/ml as a 30 x stock, which was then diluted to 1 x with SGF. Acidified bovine hemoglobin (2% mass to volume) was prepared and digestions to evaluate the labeled pepsin activity were performed in triplicate (1.25 ml per tube).

The amount of pepsin powder used to prepare SGF was calculated from the specific activity labeled on the product as 2,810 units /mg solid pepsin product. One unit activity is defined as a change in $A_{280\,nm}$ of 0.001 at 37 °C, measured as trichloracetic acid (TCA)-soluble products using bovine hemoglobin as the substrate. The assay was designed for fixed volumes and a fixed amount of test protein so the amount of pepsin diluted in SGF is adjusted to provide the appropriate ratio of 10 units of pepsin activity per microgram of test protein in the digestion mixture. The appropriate amount of solid pepsin was added to SGF to provide 4,000 units (for 10 units per microgram test protein) and 400 units (for 1 unit per microgram test protein) per

1.52 mL of SGF, respectively. The pepsin/SGF reaction mixture was preheated to 37°C in a water bath before adding 80 microliters of test protein (5 mg/mL) for a total volume of 1.6 mL, providing 10 units and 1 unit per microgram test protein, respectively.

Once the pre-heated (37°C) test protein solution was mixed with pre-heated pepsin-SGF, equal volume samples were withdrawn at predetermined times (between 0.5 and 60 minutes) and added to sample tubes containing neutralization (carbonate buffer, pH 11) and denaturing reagents (reducing Laemmli buffer) and immediately heated to 95°C, which stopped the digestion. Samples were then cooled in an ice-bath and then heated to > 85°C before running in SDS-PAGE. All samples from a single digestion were applied to wells of the same SDS-PAGE gel along with molecular weight markers, undigested test protein equivalent to the initial undigested test protein sample and a 10% test protein sample and pepsin alone (to assess pepsin stainable protein bands). Samples were separated by electrophoresis, stained with Coomassie Brilliant Blue R-250 solution (at least 6 hours), destained in R-250 destaining solution and water, and the stained gels captured using a Kodak Gel Logic 440 system (Carestream, Rochester, NY). The stability of the protein was defined as the time required to achieve 90% digestion, which was estimated based on the shortest time-digested sample with a band intensity equal to, or less than the 10% undigested standard well (P1/10). Any new bands above approximately 3,000 MW, which were generated as intermediate products of digestion, were noted as stable (or partially stable) intermediate proteolytic fragments and were considered based on stability. If those bands were also in the pepsin only controls (time 0 and time 60 mins), they were discounted as being from pepsin. Otherwise they would be analyzed by proteomic methods to determine whether they were fragments of the test protein.

Proteins with more than 10% stainable full-length protein band remaining at 60 minutes were considered stable. Proteins reduced to < 10% stainable band at 5 to 30 minutes were considered of intermediate stability. Proteins reduced to < 10% stainable band by 2 minutes were considered labile (rapidly digested).

3.1 Justification for Selection of the Test System

In vitro digestion models are used commonly to assess the digestibility of ingested substances. Previous studies have used this simple, *in vitro* assay to evaluate potential risk of food allergy, and demonstrated that stability in pepsin is a risk factor for food allergy, which might be related to initial sensitization or to elicitation once the individual is sensitized (Astwood *et al.*, 1996 and del Val *et al.*, 1999). The FAO/WHO (2001) suggested conducting the pepsin digestion assay at pH 1.2 and pH 2.0. We have performed additional independent tests showing results were quite similar for most test proteins using pH 1.2 or 2.0 (Ofori-Ant *et al.*, 2008). In this analysis, digestion was performed at pH 2.0 as a conservative approach as some authors have claimed a lack of predictive value for the digestion assay in pepsin at pH 1.2 (Fu *et al.*, 2002; Yagami *et al.*, 2000). However, Bannon *et al.* (2002) reviewed a broad range of published representative

pepsin digestion studies and found a strong positive predictive value when comparing the stability of allergenic and non-allergenic dietary proteins. As defined by Codex (2003), this assay, which measures the resistance of a test protein to proteolysis in a test tube assay. It is not meant to be a stand-alone determinant in evaluating the potential allergenicity of proteins introduced into GM crops and is not intended to predict the fate of proteins in the digestive tract of consumers. The results are to be judged in a weight of evidence approach which should also include history of safe use, sequence identity matches to known allergens and abundance of the protein in food material.

3.2 Experimental Controls

Controls in this study were meant to ensure assay reliability and include:

- Measurement of the activity of pepsin in SGF.
- Evaluation of the sensitivity of the staining properties of the test protein from serially diluted samples, in a separate, but similar SDS-PAGE gel.
- Inclusion of samples of pepsin without test protein at times zero and 60 minutes to determine whether any stainable protein bands observed in digestion samples with test protein are from the test protein, contaminants in pepsin or from pepsin autocatalysis.
- Inclusion of protein in SGF without pepsin at times zero and over 60 minutes to evaluate the effect of acid and heat alone.

3.3 Sample Retention

Samples of test protein and digested samples were numbered to distinguish assay time points and assay replicates by date. Residual samples were stored at -20°C and will be discarded approximately six months after the completion of the study.

4. Detailed Study Methods. This study evaluated the stability of recombinant leghemoglobin from Glycine *max* in pepsin at pH 2.0. A number of control steps were performed to ensure study validity. A detailed description of the study is presented here. Laboratory records and protocols are on file in the Goodman laboratory, Dept. of Food Science & Technology, University of Nebraska, Lincoln, USA.

4.1 Verification of Detection System Specificity and Sensitivity. A dilution series of sample was prepared with sample quantities loaded in SDS-PAGE gel using 1 x reducing Laemmli buffer, covering the range representing 200% total protein per well (296 µg/ml of total protein) down to 2.5 % (3.7 µg/ml of total protein. Bio-Rad precision plus protein MW markers were applied to separate lanes. Following electrophoresis, the gels were stained with Coomassie Brilliant Blue for at least 2 hours. The gels were destained 3 times with destaining solution and water until the background was clear. The image was captured using the Kodak Gel Logic 440 Image Station.

4.2 Preparation of SGF Plus Pepsin. The simulated gastric fluid (SGF) reaction buffer was prepared by adding 122.8 mg of NaCl to 59.94 mL of distilled water. The pH of the solution was adjusted to pH 2.0 using approximately 60 µl of 6.1 N HCl and water. The HCl content was approximately 0.084 N, and the salt concentration was 35 mM NaCl. The certified activity of pepsin A from Worthington was used to calculate the amount of solid pepsin that was dissolved in 1.52 mL of SGF. For this lot, the certified value was 2810 units per mg of pepsin solid material. The first target was 4,000 units of activity per 1.52 ml solution which is 10 units pepsin activity per 1 µg tested protein. Based on the Worthington analysis, the concentration of pepsin A used in the assay was 0.93 mg/ml, which is 0.093 g of solid pepsin adding to 100 ml of SGF. The second target was 400 units of activity per 1.52 ml solution which is 1 unit activity per 1 µg tested protein. The concentration used was 0.093 mg/ml, which is 10 dilution of the previously made solution with SGF. After thoroughly dissolved and mixed, the pepsin solutions were stored at 4°C and assayed for activity and used within 24 hours.

4.3 Pepsin Activity Assay. Each time SGF plus pepsin was prepared for a digestion assay; the activity of the pepsin and the digestion assay were both completed within 24 hours. The purpose of performing the activity assay was to ensure that the pepsin was active within a pre-defined range around the certified claim of activity by Worthington. This product has a labeled activity of 2,810 units per mg of solid material. The activity assay we used was similar, but not identical to that used by Worthington. The tolerance was +/- 23% of the target units per mg compared to the Worthington certified claim. The SGF plus pepsin was freshly prepared and stored at 4°C just before use, and then warmed to 37°C before the addition of the target protein. The procedure was performed as follows:

4.3.1 A solution of 2% acidified bovine hemoglobin (Hb) was prepared by dissolving 0.5 g of hemoglobin (Sigma # H2625) in 20 mL of distilled water, then mixing with 5 mL of 300 mM HCl.

4.3.2 Three polypropylene screw-top centrifuge tubes were labeled as Test (#1-3), three were labeled as Blank (#1-3), each received 1.25 mL of 2% acidified Hb and all were preheated to 37°C for 10 min.

4.3.3 At a timed interval (~ 1 min.), each of the test tubes in turn received 0.25 mL of SGF plus pepsin, was mixed by gentle vortex and returned to the incubator. As each test tube reached 10 min. incubation time, 2.5 mL of 5% TCA (Sigma 6.1 N product T0699, diluted 1:20 with distilled water) was added to stop the reaction, the tube was mixed briefly by multiple inversion and then placed on ice to cool down. Then insoluble material (undigested hemoglobin) was removed using syringes (LuerLok BD 309646, 5 ml) and syringe filters (Corning Incorporated, 0.45 µm PTFE, product #431220).

4.3.4 Blank tubes were interspersed with the Test tubes. Blank tubes (with 1.25 mL of Hb) received 2.5 mL of 5% TCA, multiple inversion, then 0.25 mL of SGF plus

pepsin. After 10 min incubation time, these tubes were also placed on ice and then filtered to remove insoluble material.

4.3.5 The absorbance at 280 nm was measured on a spectrophotometer (Spectronic Genesys 5, MILTON ROY). The activity units of pepsin per mL were calculated as the mean net absorbance (A280 nm Hb – A280 controls) multiplied by a conversion factor of 1,000 to yield units of activity per mg of solid pepsin.

4.4 Control Protein Digestions (Hemoglobin, BSA and OVA). Bovine hemoglobin, bovine serum albumin (BSA) and chicken ovalbumin (OVA) digestion assays were tested as control proteins to verify the appropriate activity of the test system.

4.5 Test Protein Digestion. The Soy Leghemoglobin (LegHb) concentration within the Soy Leghemoglobin Preparation was estimated by Impossible Foods as 79 mg/ml of total protein containing 66% LegHb, and was measured as 79.94 mg/ml of total protein in our laboratory. We have used 79.94 mg/ml value for calculating concentrations. Protein solutions were aliquoted and kept at −20 °C until immediately before use.

4.5.1 Sample tube preparation. 1.5 mL centrifuge tubes were labeled as P1/10, P0, P60, D0, D0.5, D2, D5, D10, D20, D30, D60, E0, E60.

4.5.2 70 µL of pepsin quenching solution (carbonate buffer) and 70 µL of 5X Laemmli, reducing buffer were added to each tube in 4.4.1.

4.5.3 An aliquot of hemoglobin in a tube labeled as P, was prepared.

4.5.3 $P_{1/10}$: 190 µL of SGF plus pepsin was added, quick heated at 85°C, then 10 µL 1/10 diluted hemoglobin solution was added. Solution was vortexed and then heated at 85°C for 10 min.

4.5.4 Label a tube P_{mx} (no pepsin, protein control): 80 µL out of tube P and then 1.52 mL SGF were added and mixed.

4.5.4.1 Immediately 200 µL into the P0 tube were removed, mixed and heated at 85°C for 10 min.

4.5.4.2 After 60 minutes at 37°C water bath, 200 µL into the P60 tube were removed, mixed and heated at 85°C for 10min.

4.5.5 Label a tube E_{mx} (pepsin enzyme, no protein control): 80 µL distilled water were added to 1.52 mL SGF plus pepsin, and then were mixed.

4.5.5.1 Immediately 200 µL into the E0 tube were removed, mixed and heated at 85°C for 10 min.

4.4.5.2 After 60 minutes at 37°C water bath, 200 µL into the E60 tube were removed, mixed and heated at 85°C for 10 min.

4.5.6 Label a tube D_{mx} (digestion mixture): 80 µL out of tube P was added to 1.52 mL SGF plus pepsin and mixed, then placed in 37°C water bath.

4.5.6.1 At 0.5, 2, 5, 10, 20, 30, 60 min internals, 200 µL of digestion mixture were withdrawn into D0.5, D2, D5, D10, D20, D30, D60 quenching tubes. (e.g.

D0.5 at 30 sec., D2 at 2 min), each sample tube was heated to 85°C for 10 min.

4.5.7 P_0: 190 µL of SGF plus pepsin was added, quick heated at 85°C, then 10 µL out of tube P was added. Solution was vortexed and then heated at 85°C for 10 min.

4.6 SDS-PAGE Gel. All samples on any one gel were from a single digestion experiment. Novex 10-20% tris-glycine gels were used with SDS-PAGE buffer.

4.6.1 10 µL of each sample tube was loaded per well, containing 1.47 µg of starting LegHb per well except in wells for $P_{1/10}$ tube which was 0.147 µg.

4.6.2 4 µL of pre-stained precision plus protein™ Dual Xtra Standards molecular weight marker proteins were loaded in the outer two wells.

4.6.3 Electrophoresis was accomplished at a constant 150 voltage.

4.6.4 Gels for staining were stained for a minimum of 6 hours in Coomassie Brilliant Blue as detailed by Bio-Rad, then destained for at least 30 min in destaining solution and water.

4.7 Image Analysis. The destained gels were visualized in a Gel Logic 440 Image Station under white light trans-illumination. The image was captured and the image intensity adjusted to optimum background and band intensities. The raw image was saved as an archival file.

4.7.1 The molecular weight of the hemoglobin, BSA, ovalbumin, LegHb and any resulting degradation band that was not in the pepsin only lane was noted.

4.7.2 The 10% control band (P1/10) was used as the standard for comparison of all digested samples on a given gel.

4.7.3 The first time point the digested band appeared to be less than the10% concentrated sample was used to estimate the time to achieve 90% digestion.

4.8 Proteomic LC-MS Identity of Bands from *Pichia pastoris*. Impossible Foods performed LC-MS/MS analysis of 10 faint bands that were visible in Figure 10 a and b, which corresponded to proteins from *Pichia pastoris* that were ≥1% of the Soy Leghemoglobin Preparation total protein fraction. All of the *Pichia* protein bands were digested and no longer visible in Fig. 9 and 10a), demonstrating they are rapidly digested.

5. Results & Discussion

5.1 Limit of Detection. The stained gel of the dilution series of total protein (Figure 1) demonstrated a clear pattern of reduced intensity of stained bands with each step in the dilution series. The minimum amount of protein that was detected was 0.075 µg of total protein which contains 0.049 µg of LegHb. Based on these data, the limit of detection was approximately 5% of total protein which contains 3.3% as LegHb at 100% loaded in the digestion samples. This level of sensitivity was clearly sufficient to detect 10% residual of hemoglobin or any other protein in the digest.

5.2 Pepsin Activity. The certified activity of the lot of pepsin from Worthington used in this study was labeled as 2,810 units per mg of solid and was tested 2826 units per mg right before the digestion assays were performed.

5.3 Control Substance Digestion Results. Stained gels of digestion tests of control substance hemoglobin, BSA and ovalbumin (Figure 2 - 7) demonstrated that at both ratio of 10 units and 1 unit of pepsin activity per 1 μg of test protein, BSA and hemoglobin were digested rapidly within the SGF plus pepsin test system and that ovalbumin was stable with more than 10% visually stainable full-length protein band remaining at 60 minutes. However, BSA left residual pepsin resistant fragments at a low MW, which was more pronounced with a ratio of 1 unit activity per microgram of protein, compared to 10 units per microgram. At the ratio of 10 units of pepsin activity per 1 μg of test protein, OVA was mostly digested to a stable fragment just below the MW of pepsin, between 20 and 60 minutes. However, when the activity ratio was reduced to 1 unit of pepsin activity per 1 μg of test protein, OVA was markedly more stable, with little digestion. These results with 10 units with OVA and BSA are consistent with results from previous tests (Ofori-Anti, A.O. 2008), which demonstrates the reproducibility of this SGF plus pepsin test system.

5.4 Leghemoglobin Protein Digestion Results. Two representative stained gels of digestion experiments of LegHb at pH 2.0 at the ratio of 10 units and 1 unit of pepsin activity per 1 μg of test protein (Figure 8-9) demonstrated that at both ratios, the LegHb protein was stable in acid alone for 60 minutes (lane 3), but rapidly digested by pepsin in 2 minutes (lane 5) to below the visible band intensity of the quenched pepsin 10% LegHb control (P1/10 control in lane 13).

5.5 Pichia pastoris proteins. Gels in Figures 8, 9 and 10, lanes 1-3 have several faint bands showing between 10 kDa, and 250 kDa, which were identified through mass spectrometry by Impossible Foods as proteins from the host, *Pichia pastoris*. The sequences of the proteins have been evaluated by bioinformatics to evaluate sequence identity matches with known allergens and toxins. They include: alpha aminoadipate reductase (1400 aa), cobalamin-independent methionine synthase (768 aa), aconitase (780 aa), transketolase (679 aa), glycerol kinase (621 aa), catalase A (510 aa), GAPDH (504 aa), hypothetical protein PAS (525 aa), mitochondrial aldehyde dehydrogenase (501 aa), delta-aminevulinate dehydrogenase (341 aa), mitochondrial alcohol dehydrogenase III (350 aa), malate dehydrogenase (342 aa), putative protein of unknown function (328 aa), triose phosphate isomerase (248 aa), hypothetical protein-cyclophilin (161 aa), cytosolic superoxide dismutase (154 aa) and mitochondrial ATPase inhibitor (84 aa). These stained protein bands were rapidly digested to the point of being invisible at time 0.5 minutes or 2 minutes (lanes 4 and 5 in the gels shown in Figures 8, 9 and 10).

6. Conclusions

The results of this study demonstrated that the LegHb protein and the *Pichia pastoris* proteins within the Soy Leghemoglobin Preparation were rapidly digested after incubation in SGF plus pepsin at 37°C, at both ratio of 10 units and 1 unit of pepsin activity per 1 µg of total protein, both with more than 90% digested within 2 minutes based on Coomassie Blue staining detection.

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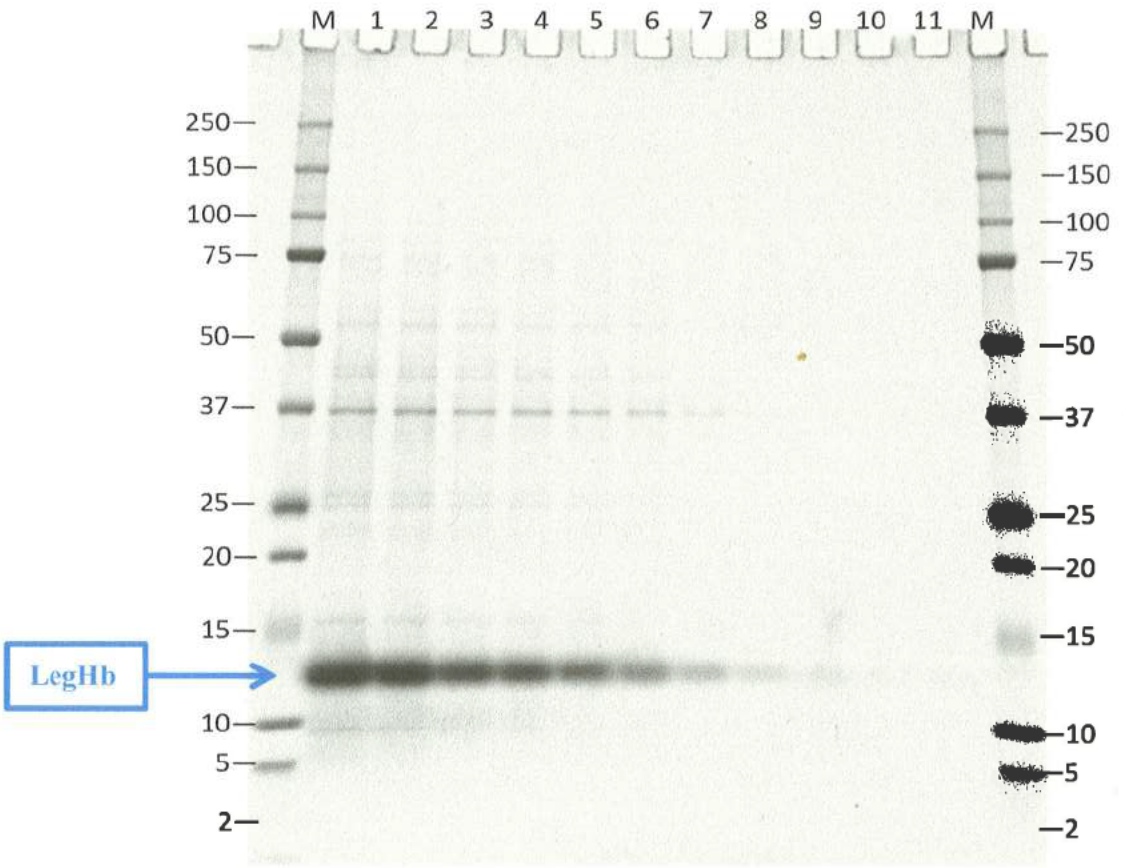

Figure 1. Coomassie Brilliant Blue Stained SDS-PAGE Gel Showing the Serial Dilution of Soy Leghemoglobin Preparation Starting from 200% of Total Protein. Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a glycine buffered gel.

Lane	Description	Protein Content
1	200% Total protein	2.96 µg (1.95 µg LegHb)
2	150% Total protein	2.21 µg (1.46 µg LegHb)
3	100% Total protein	1.47 µg (0.97 µg LegHb)
4	80% Total protein	1.18 µg (0.78 µg LegHb)
5	60% Total protein	0.88 µg (0.58 µg LegHb)
6	40% Total protein	0.59 µg (0.39 µg LegHb)
7	20% Total protein	0.29 µg (0.19 µg LegHb)
8	10% Total protein	0.15 µg (0.097 µg LegHb)
9	5% Total protein	0.075 µg (0.049 µg LegHb)
10	2.5% Total protein	0.037 µg (0.024 µg LegHb)
M	Molecular weight Marker	na

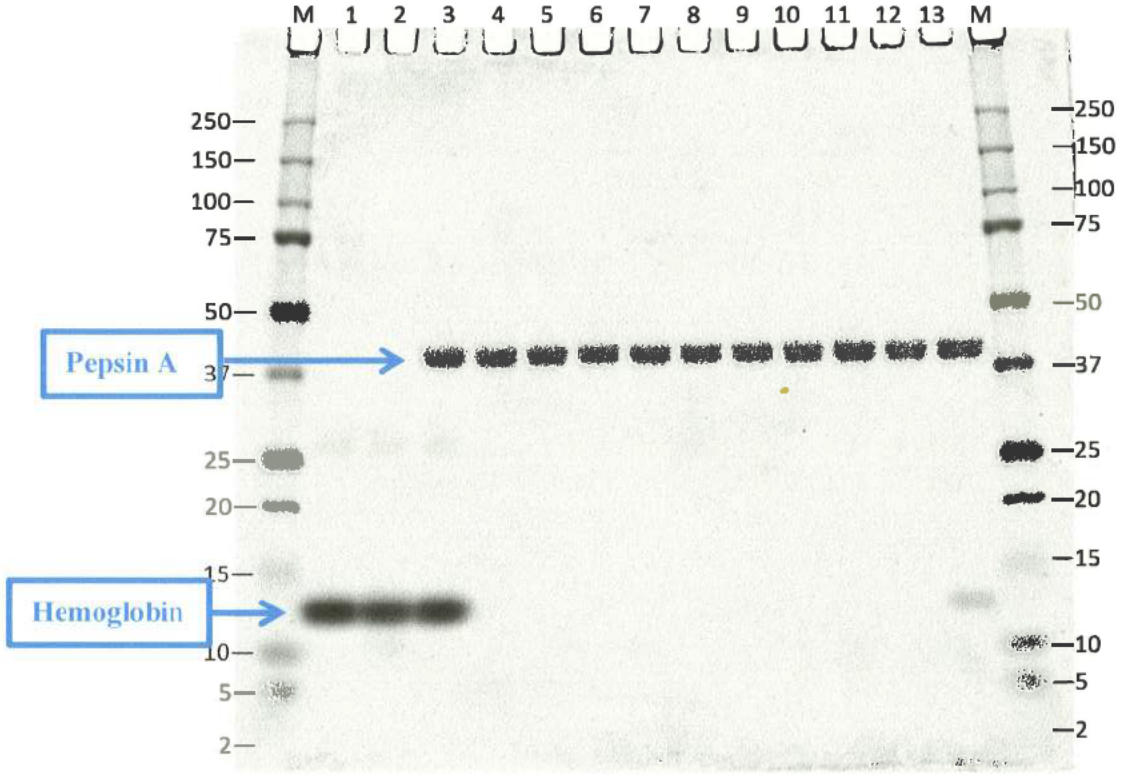

Figure 2. Coomassie Brilliant Blue Stained SDS-PAGE Gel Showing the Digestion of Bovine Hemoglobin in Simulated Gastric Fluid at the ratio of 10 Units per µg Protein (pH 2.0). Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a glycine buffered gel. Hemoglobin was loaded 1.47 µg per lane based on pre-digestion concentration (pH 2.0).

Lane	Description	Incubation time	
M	Molecular weight Marker	na	
1	Experimental control: Hemoglobin (P0)	0	min
2	Experimental control: Hemoglobin (P60)	60	min
3	Hemoglobin in SGF, (D0)	0	min
4	Hemoglobin in SGF, (D0.5)	0.5	min
5	Hemoglobin in SGF, (D2)	2	min
6	Hemoglobin in SGF, (D5)	5	min
7	Hemoglobin in SGF, (D10)	10	min
8	Hemoglobin in SGF, (D20)	20	min
9	Hemoglobin in SGF, (D30)	30	min
10	Hemoglobin in SGF, (D60)	60	min
11	Experimental control: Pepsin (E0)	0	min
12	Experimental control: Pepsin (E60)	60	min
13	10% Hemoglobin with quenched pepsin (P1/10)	0	min

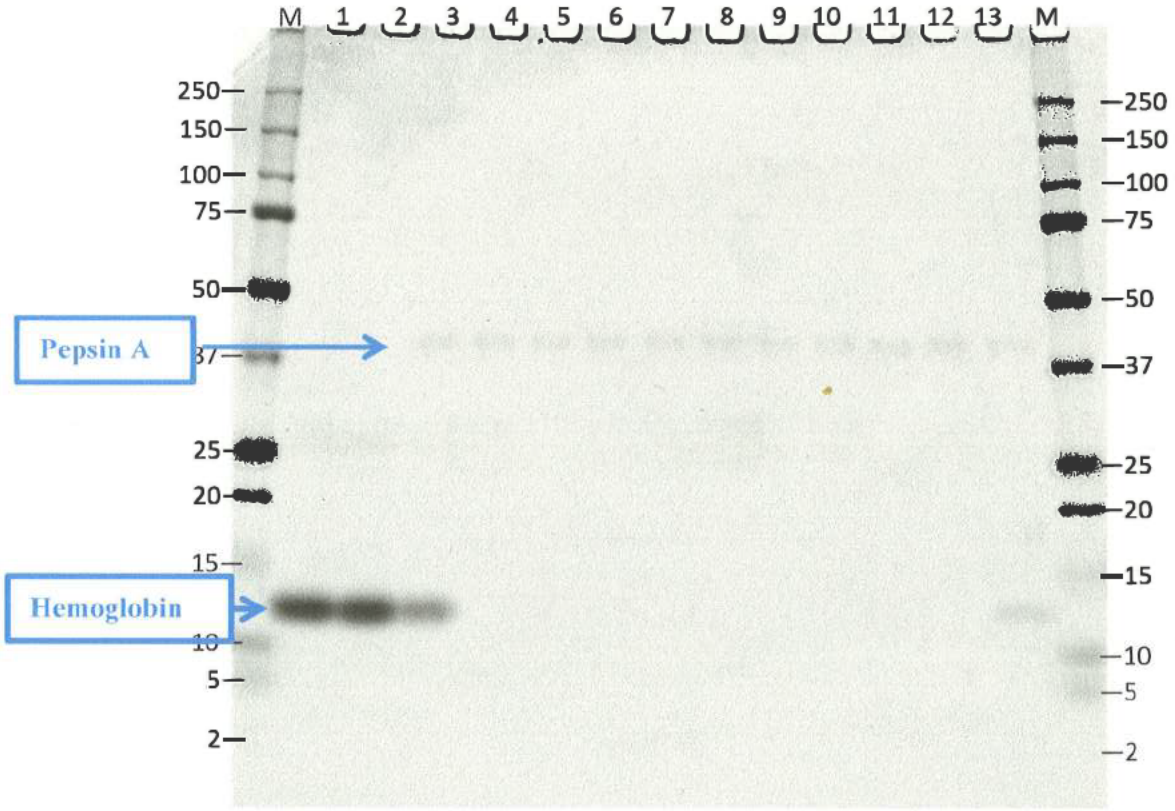

Figure 3. Coomassie Brilliant Blue Stained SDS-PAGE Gel Showing the Digestion of Bovine Hemoglobin in Simulated Gastric Fluid at a Ratio of 1 units per µg (pH 2.0). Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a glycine buffered gel. Hemoglobin was loaded 1.47 µg per lane based on pre-digestion concentration (pH 2.0).

Lane	Description	Incubation time	
M	Molecular weight Marker	na	
1	Experimental control: Hemoglobin (P0)	0	min
2	Experimental control: Hemoglobin (P60)	60	min
3	Hemoglobin in SGF, (D0)	0	min
4	Hemoglobin in SGF, (D0.5)	0.5	min
5	Hemoglobin in SGF, (D2)	2	min
6	Hemoglobin in SGF, (D5)	5	min
7	Hemoglobin in SGF, (D10)	10	min
8	Hemoglobin in SGF, (D20)	20	min
9	Hemoglobin in SGF, (D30)	30	min
10	Hemoglobin in SGF, (D60)	60	min
11	Experimental control: Pepsin (E0)	0	min
12	Experimental control: Pepsin (E60)	60	min
13	10% Hemoglobin with quenched pepsin (P1/10)	0	min

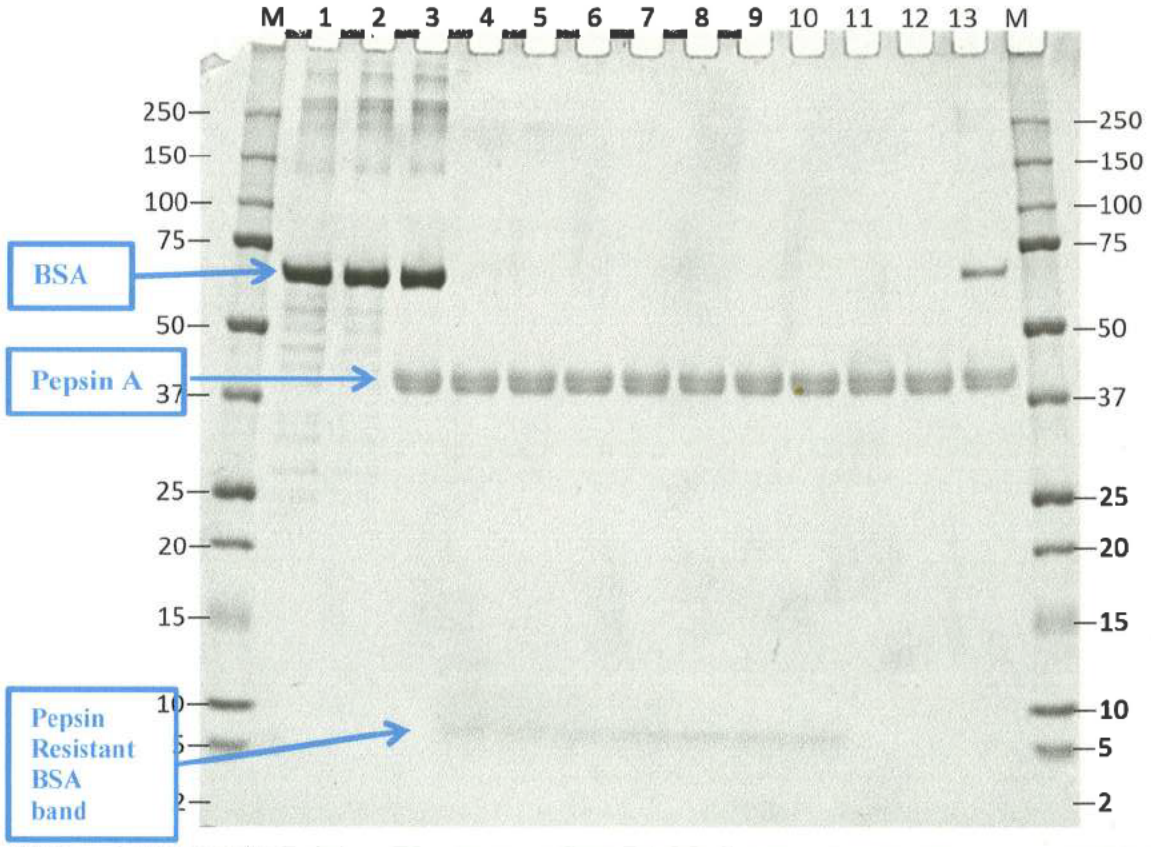

Figure 4. Coomassie **Brilliant Blue Stained SDS-PAGE Gel Showing the Digestion of BSA in Simulated Gastric Fluid at the ratio of 10 units per µg protein (pH 2.0).** Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a glycine buffered gel. BSA was loaded 1.47 µg per lane based on pre-digestion concentration (pH 2.0).

Lane	Description	Incubation time	
M	Molecular weight Marker	na	
1	Experimental control: BSA (P0)	0	min
2	Experimental control: BSA (P60)	60	min
3	BSA in SGF, (D0)	0	min
4	BSA in SGF, (D0.5)	0.5	min
5	BSA in SGF, (D2)	2	min
6	BSA in SGF, (D5)	5	min
7	BSA in SGF, (D10)	10	min
8	BSA in SGF, (D20)	20	min
9	BSA in SGF, (D30)	30	min
10	BSA in SGF, (D60)	60	min
11	Experimental control: Pepsin (E0)	0	min
12	Experimental control: Pepsin (E60)	60	min
13	10% BSA with quenched pepsin (P1/10)	0	min

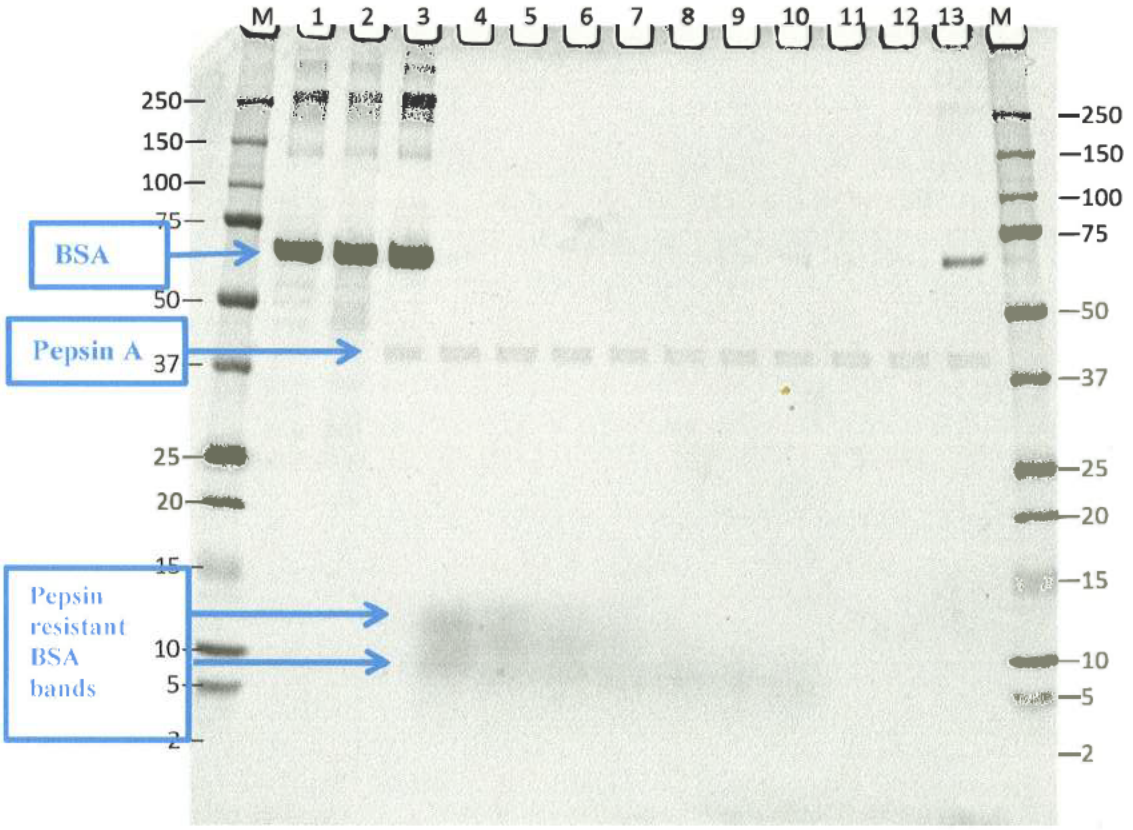

Figure 5. Coomassie Brilliant Blue Stained SDS-PAGE Gel Showing the Digestion of BSA in Simulated Gastric Fluid at the Ratio of 1 Unit per µg Protein (pH 2.0). Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a glycine buffered gel. BSA was loaded 1.47 µg per lane based on pre-digestion concentration (pH 2.0).

Lane	Description	Incubation time	
M	Molecular weight Marker	na	
1	Experimental control: BSA (P0)	0	min
2	Experimental control: BSA (P60)	60	min
3	BSA in SGF, (D0)	0	min
4	BSA in SGF, (D0.5)	0.5	min
5	BSA in SGF, (D2)	2	min
6	BSA in SGF, (D5)	5	min
7	BSA in SGF, (D10)	10	min
8	BSA in SGF, (D20)	20	min
9	BSA in SGF, (D30)	30	min
10	BSA in SGF, (D60)	60	min
11	Experimental control: Pepsin (E0)	0	min
12	Experimental control: Pepsin (E60)	60	min
13	10% BSA with quenched pepsin (P1/10)	0	min

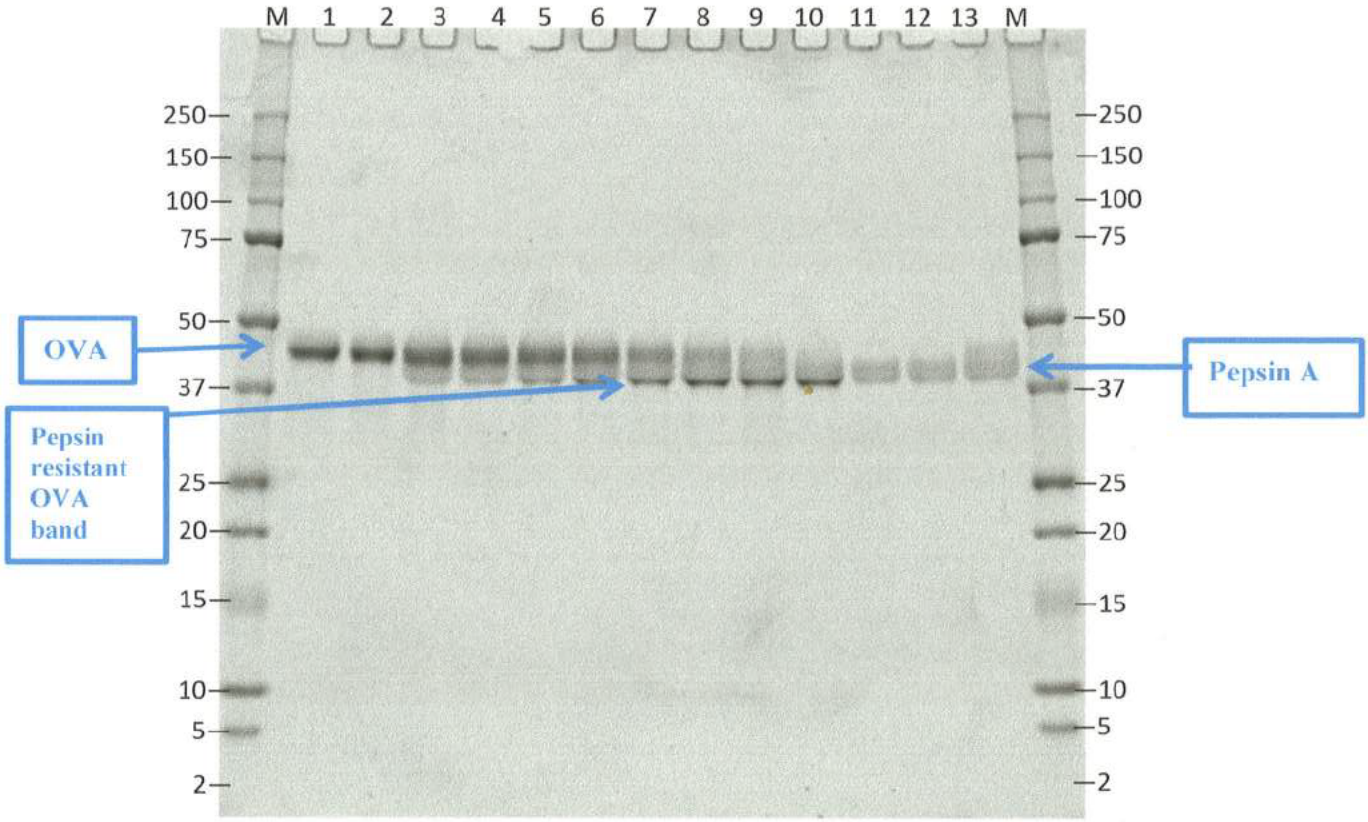

Figure 6. Coomassie Brilliant Blue Stained SDS-PAGE Gel Showing the Digestion of OVA in Simulated Gastric Fluid at the Ratio of 10 Units per µg Protein (pH 2.0). Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a glycine buffered gel. OVA was loaded 1.47 µg per lane based on pre-digestion concentration (pH 2.0).

Lane	Description	Incubation time	
M	Molecular weight Marker	na	
1	Experimental control: OVA (P0)	0	min
2	Experimental control: OVA (P60)	60	min
3	OVA in SGF, (D0)	0	min
4	OVA in SGF, (D0.5)	0.5	min
5	OVA in SGF, (D2)	2	min
6	OVA in SGF, (D5)	5	min
7	OVA in SGF, (D10)	10	min
8	OVA in SGF, (D20)	20	min
9	OVA in SGF, (D30)	30	min
10	OVA in SGF, (D60)	60	min
11	Experimental control: Pepsin (E0)	0	min
12	Experimental control: Pepsin (E60)	60	min
13	10% OVA with quenched pepsin (P1/10)	0	min

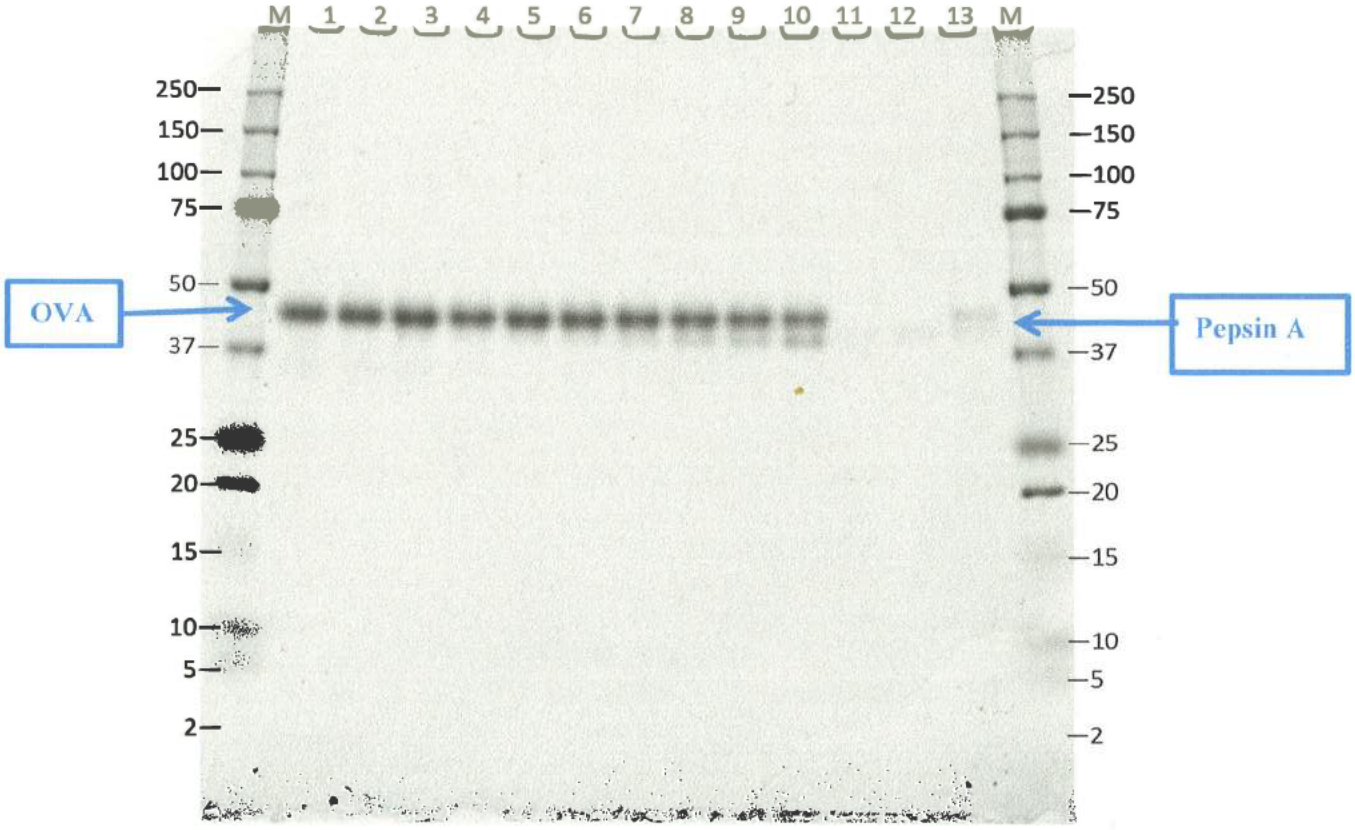

Figure 7. Coomassie Brilliant Blue Stained SDS-PAGE Gel Showing the Digestion of OVA in Simulated Gastric Fluid at the Ratio of 1 Unit per µg Protein (pH 2.0). Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a glycine buffered gel. OVA was loaded 1.47 µg per lane based on pre-digestion concentration (pH 2.0).

Lane	Description	Incubation time	
M	Molecular weight Marker	na	
1	Experimental control: OVA (P0)	0	min
2	Experimental control: OVA (P60)	60	min
3	OVA in SGF, (D0)	0	min
4	OVA in SGF, (D0.5)	0.5	min
5	OVA in SGF, (D2)	2	min
6	OVA in SGF, (D5)	5	min
7	OVA in SGF, (D10)	10	min
8	OVA in SGF, (D20)	20	min
9	OVA in SGF, (D30)	30	min
10	OVA in SGF, (D60)	60	min
11	Experimental control: Pepsin (E0)	0	min
12	Experimental control: Pepsin (E60)	60	min
13	10% OVA with quenched pepsin (P1/10)	0	min

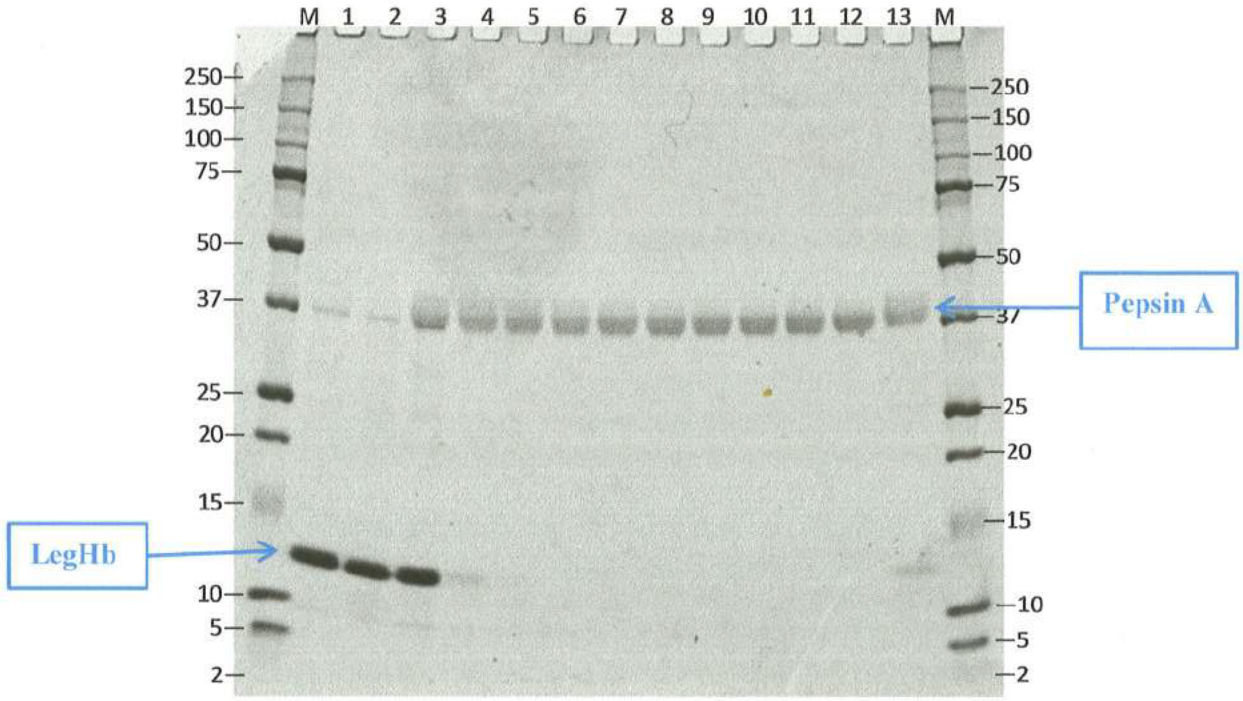

Figure 8. Coomassie Brilliant Blue Stained SDS-PAGE Gel Showing the Digestion of Soy Leghemoglobin Preparation in Simulated Gastric Fluid at the Ratio of 10 Units per µg Protein (pH 2.0). Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a glycine buffered gel. LegHb was loaded 1.47 µg per lane based on pre-digestion concentration (pH 2.0).

Lane	Description	Incubation time	
M	Molecular weight Marker	na	
1	Experimental control: LegHb (P0)	0	min
2	Experimental control: LegHb (P60)	60	min
3	LegHb in SGF, (D0)	0	min
4	LegHb in SGF, (D0.5)	0.5	min
5	LegHb in SGF, (D2)	2	min
6	LegHb in SGF, (D5)	5	min
7	LegHb in SGF, (D10)	10	min
8	LegHb in SGF, (D20)	20	min
9	LegHb in SGF, (D30)	30	min
10	LegHb in SGF, (D60)	60	min
11	Experimental control: Pepsin (E0)	0	min
12	Experimental control: Pepsin (E60)	60	min
13	10% LegHb with quenched pepsin (P1/10)	0	min

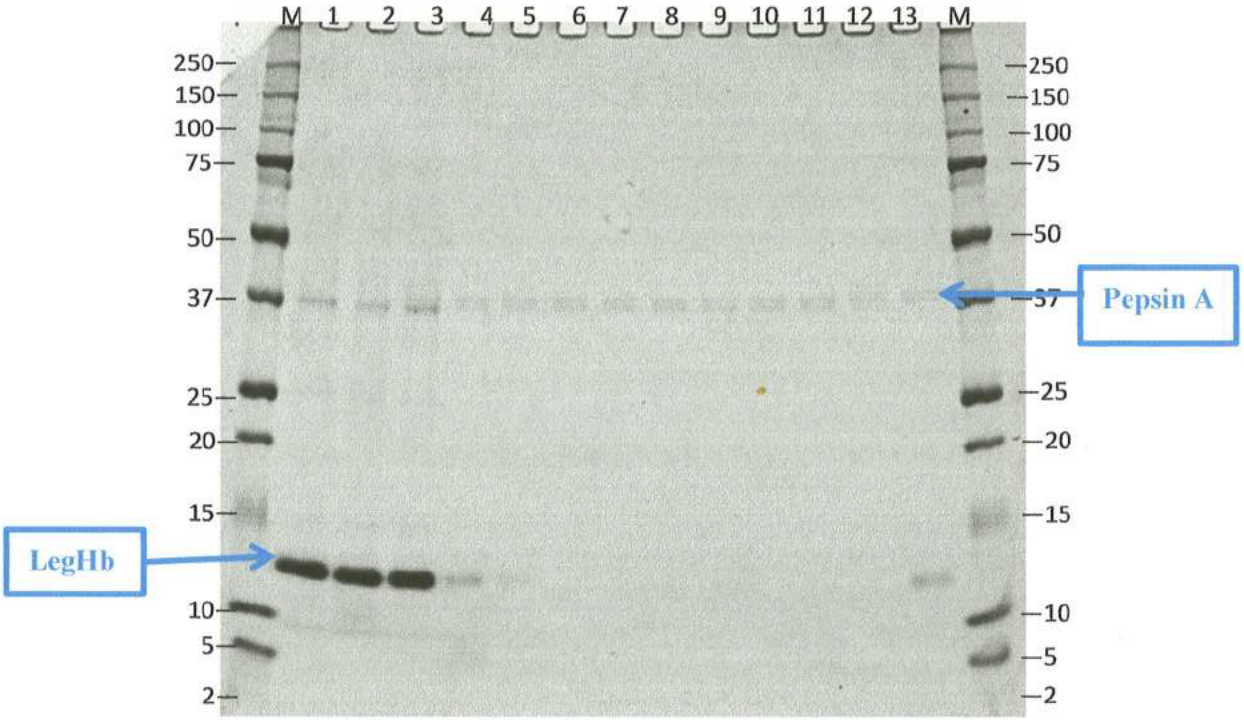

Figure 9. Coomassie Brilliant Blue Stained SDS-PAGE Gel Showing the Digestion of Soy Leghemoglobin Preparation in Simulated Gastric Fluid at the Ratio of 1 Unit per µg Protein (pH 2.0). Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a glycine buffered gel. LegHb was loaded 1.47 µg per lane based on pre-digestion concentration (pH 2.0).

Lane	Description	Incubation time	
M	Molecular weight Marker	na	
1	Experimental control: LegHb (P0)	0	min
2	Experimental control: LegHb (P60)	60	min
3	LegHb in SGF, (D0)	0	min
4	LegHb in SGF, (D0.5)	0.5	min
5	LegHb in SGF, (D2)	2	min
6	LegHb in SGF, (D5)	5	min
7	LegHb in SGF, (D10)	10	min
8	LegHb in SGF, (D20)	20	min
9	LegHb in SGF, (D30)	30	min
10	LegHb in SGF, (D60)	60	min
11	Experimental control: Pepsin (E0)	0	min
12	Experimental control: Pepsin (E60)	60	min
13	10% LegHb with quenched pepsin (P1/10)	0	min

Figure 10 a)

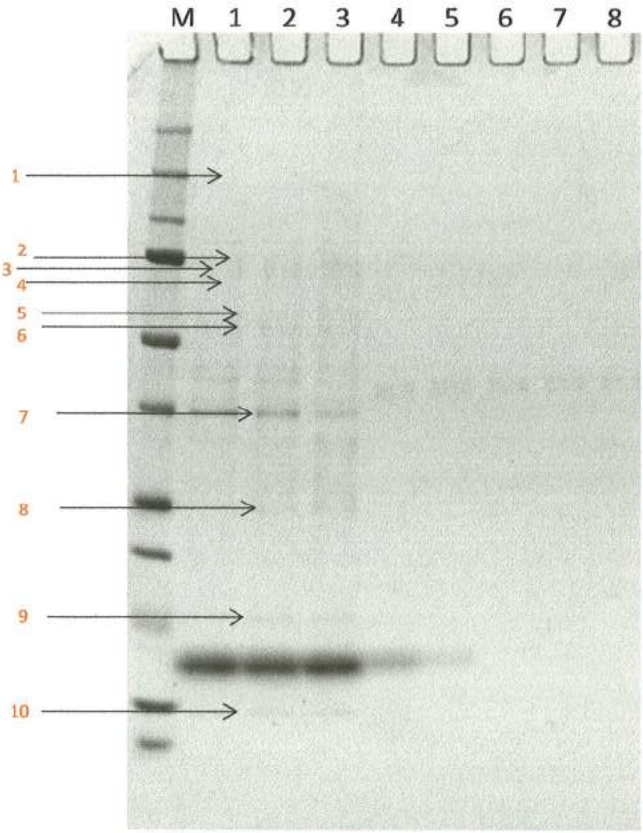

Figure 10 b)

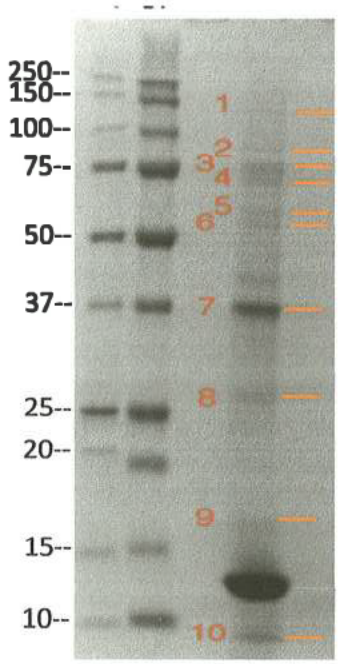

Figure 10. Coomassie Brilliant Blue Stained SDS-PAGE Gel of Soy Leghemoglobin Preparation with 1 Unit pepsin activity per µg Protein (pH 2.0) from Figure 9 (left panel, 10 a), and protein identity gel from Impossible Foods (right panel 10 b). Coomassie stained gels showing the 10 bands of *P. pastoris* proteins that were identified by LC-MS/MS. Note that all 10 bands of *Pichia* proteins are visible at time zero, but not at time 30 seconds of digestion in pepsin (lane 4 of figure 10 a).