APPENDIX I
MORI SILK *IN VITRO* DIGESTIBILITY STUDY IN HUMAN SIMULATED GASTRIC FLUID

STUDY TITLE

Silk Fibroin: *in vitro* digestibility study in human simulated gastric fluid (pH 2.0) at 10 units per µg test protein

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STUDY COMPLETED ON

12 February 2020

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LABORATORY STUDY ID

20200212 – REG – Silk Fibroin Digestion

Statement of No Data Confidentiality

No claim of confidentiality is made for any information contained in this document. I acknowledge that information not designated as within the scope of FIFRA Section 10(d)(l)(A), (B), or (C) and which pertains to a registered pesticide is not entitled to confidential treatment and may be released to the public, subject to the provisions regarding disclosure to multinational entities under FIFRA section 10(g).

Company

Company Agent:

[redacted]

Laith M. Abu-Taleb, Esq.
Cambridge Crops, Inc.

March 18, 2020
Date

These Data May Be Considered Confidential In Countries Outside The United States.

GLP 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. However, raw data and bioinformatics comparisons were archived in PDF format and remain at Cambridge Crops, Inc.

Applicant/Sponsor

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Date: March 18, 2020

Expert Review Statement

I assisted the Cambridge Crops team with the design of the study outlined in this report. Together, we worked on developing the protocols based off of the Ofori-Anti *et al.* (2008) publication. I did not conduct the study in my laboratory. I did observe the results and was given a chance to view the SDS-PAGE gel as shown in the Figures in Section 8. I have reviewed this paper and agree with the conclusions made by the Cambridge Crops team.

Advisor: [redacted]

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Date: 18 March 2020

Study Number:	20200212 – REG – Silk Fibroin Digestion
Title:	Silk Fibroin: *in vitro* digestibility study in human simulated gastric fluid (pH 2.0) at a ratio of 10 units per µg
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Records Retention:	All study specific raw data and a copy of the final report will be retained at Cambridge Crops, Inc.

Signature of Final Report Approval:

Principal Investigator: Sezin Yigit, Ph.D.

03/18/2020
Date

SUMMARY

The silk fibroin protein used in this study was produced and supplied by Cambridge Crops, Inc. of Somerville, MA. The protein is derived from silkworm (*Bombyx mori*) cocoons and is comprised of three chains: the light chain, heavy chain (UniProt P05790), and glycoprotein P25. The silk fibroin test material used in this study was provided in its solid, powdered form.

The silk fibroin was subjected to digestion based on the protocol in Ofori-Anti *et al.* (2008)[1] 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 compared a non-digested sample. A limit of detection study was performed prior to digestion to ensure that 10% residual protein was detectable using SDS-PAGE and Coomassie blue staining. Due to the methods used during processing, the silk fibroin used in these studies appears as smear on SDS-PAGE. Pepsin was diluted in simulated gastric fluid (SGF) with an adjusted pH to 2.0. The pepsin solution was tested for proteolytic activity by digestion of silk fibroin within 24 hours of each assay day. The mass ratio of pepsin to silk fibroin preparation was adjusted to achieve 10 units of pepsin activity per microgram of total protein in solution. Digestions were performed at 37°C under timed conditions. Samples of the digestion mixtures were removed and neutralized at various time points from 2 minutes 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 silk fibroin protein is rapidly digested in pepsin at pH 2.0 at a ratio of 10 units pepsin per microgram silk fibroin. The SDS-PAGE Coomassie blue gel staining method demonstrated that over 90% of the silk fibroin protein was digested in less than two minutes.

No degradation bands were found to result from digestion of the silk fibroin. Therefore, our conclusion is that silk fibroin is rapidly digested at a ratio of 10 units pepsin per microgram silk fibroin in 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 silk fibroin in pepsin.

[1] Ofori-Anti, A.O., Ariyarathna, H., Chen, L., Lee, H.L., Pramod, S.N., Goodman, R.E. (2008). Establishing objective detection limits for the pepsin digestion assay used in the assessment of genetically modified foods. *Reg. Toxicol. Pharmacol.* **52**:94-103.

TABLE OF CONTENTS

1. Introduction

Cambridge Crops, Inc. of Somerville, MA has developed a shelf-life extension technology using silk fibroin derived from the cocoons of *Bombyx mori* as the primary ingredient. Cambridge Crops performed tests in order to determine whether there is a risk of food allergy associated with the consumption of the protein. This report describes the rationale, test methods, and results pertaining to an *in vitro* digestion assay intended to provide data indicative of potential risks of food safety.

The *Codex Alimentarius* Commission guidelines for assessing the allergenicity (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 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 (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 several 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. The pure porcine pepsin protein used in this assay was purchased from VWR International, product 10791-836 (Lot # 18J2056015).

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 LDS 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 serum albumin (BSA), as described by VWR, to ensure that it is within a tolerance interval reported by VWR for that lot of enzyme. The results of our activity assay fell within the acceptance criterion of the VWR certified activity at 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 (silk fibroin 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 this, a serial dilution of test protein is run on the same SDS-PAGE and Coomassie blue staining system as the digested samples and are analyzed to evaluate a limit of detection (LOD). The LOD must be lower than 10% to perform the digestion assay. Details and results of the study are reported here.

2. Materials

2.1 Test Substance

The test substance for this study was silk fibroin isolated from the cocoons of the silkworm, *Bombyx mori*. The sample was manufactured by Cambridge Crops' production run 17-0202-042-P65.

2.2 Control Substance

The control substance for this study was bovine serum albumin (BSA), purchased from Prospec Bio (Catalog # PRO-422). BSA was used to confirm the pepsin activity indicated by the manufacturer (Difco™ Pepsin manufactured by BD Biosciences; Catalog # 215110; CAS Number 9001-75-6; purchased from VWR International).

2.3 Reference Substance

There was no reference substance used 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 Critical Analytical Reagents

- Pepsin (VWR International, Cat.# 10791-836, Lot# 18J2056015)
- SGF without pepsin: 35 mM HCl, 123 mM NaCl (adjusted to pH = 2)
- SGF with pepsin: 105 mg/mL pepsin dissolved in SGF (1.05×10^6 U/mL)
- Bovine serum albumin (BSA)
- Pepsin quenching solution: 0.7 M Na_2CO_3 (pH = 11)
- NuPAGE LDS Sample Buffer (4x) (Invitrogen, Cat.# NP0007, Lot# 2020067)
- PageRuler Prestained NIR Protein Ladder (ThermoFisher, Cat.# 26635, Lot# 00810782)
- NuPAGE 3-8% Tris-Acetate Protein Gels (Invitrogen, Cat.# EA0375BOX, Lot# 19072371)
- MOPS SDS Running Buffer (20x) (G Biosciences, Cat.# 786-926, Lot# 190511)
- InstantBlue Protein Stain (Expedeon, Cat.# ISB1L)

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 Sigma Aldrich for determining the activity of pepsin. An appropriate mass of pepsin powder was dissolved in prepared SGF, pH 2.0 to provide 2 mg/ml. Acidified bovine serum albumin (2% mass to volume) was prepared and digestions to evaluate the labeled pepsin activity were performed in triplicate.

The amount of pepsin powder used to prepare SGF was calculated from the specific activity labeled on the product as 10,000 units /mg solid pepsin product. 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 test protein. Pepsin stock is dissolved in SGF at a concentration of 105 mg/mL (1.05×10^6 U/mL). In addition, a silk fibroin stock was made at 42 mg/mL in SGF. The digestion reaction mixture was made by mixing 1.43 mL silk fibroin stock solution and 570 µL pepsin stock solution. This achieves the desired ratio of 10 units pepsin per microgram silk fibroin protein in 2 mL. The reaction mixture is placed in a preheated incubator at 37 °C and gently agitated at 120 RPM for the duration of the experiment.

Once the test protein solution was placed in the incubator (37 °C), 200 µL aliquots were withdrawn at predetermined times (t = 0, 2, 5, 10, 20, 30, 60 min.) and added to test tubes containing a mixture of 70 µL 0.7 M Na_2CO_3 (pH = 11) and 70 µL 5x LDS buffer, for a total of 340 µL. Upon addition of the test solution to the denaturing reagents (LDS buffer and Na_2CO_3), the samples are immediately heated in a water bath to 95 °C for 5 min. in order to halt digestion. Samples were allowed to cool to room temperature before running on SDS-PAGE at 350 µg/well.

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 (t = 0 and 60 min.), and pepsin alone (t = 0 and 60 min.) to asses pepsin stability throughout the duration of the assay.

Samples were separated by electrophoresis, stained with InstantBlue Protein Stain, and images were captured using a Canon PowerShot SX540 camera. 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 well in the LOD assay. 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. 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 measures the resistance of a test protein to proteolysis in a test tube system. It is not meant to be a stand-alone determinant in evaluating the potential allergenicity of proteins 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 via SDS-PAGE
- Inclusion of samples of pepsin without test protein at t = 0 and 60 min. 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.

4. Detailed Study Methods

This study evaluated the stability of silk fibroin, derived from the cocoons of *Bombyx mori*, in pepsin in SGF at pH = 2. Several control steps were performed to ensure study validity. A detailed description of the study is presented here. Laboratory records and protocols are on file at Cambridge Crops, Inc. in Somerville, MA and may be made available upon request.

4.1 Verification of Detection System and Sensitivity

A dilution series of test sample was prepared with sample quantities loaded in SDS-PAGE gel using 4x LDS buffer, covering a range from 100% total protein per well (350 µg) to 1% total protein per well (3.5 µg). Following electrophoresis, the gels were stained with InstantBlue (Coomassie) for at least 2 hours. The gels were washed with deionized water three times until the background was clear. The image was captured using Canon PowerShot SX540 camera.

4.2 Preparation of SGF Plus Pepsin

The simulated gastric fluid (SGF) was prepared by mixing 1.75 mL 1.0 M HCL with 48.25 mL diH_2O and then adding 359.4 mg NaCl. This achieves a final SGF solution concentration of 35 mM HCl and 123 mM NaCl. The activity of pepsin purchased from VWR International was verified to be 10,000 U/mg. A stock pepsin solution was prepared by adding 1.05 g pepsin to 20 mL SGF. This achieves a final pepsin stock concentration of 105 mg/mL (1.05×10^6 U/mL). 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

The activity of pepsin was verified with Bovine Serum Albumin ("BSA") to ensure the activity units claimed by the vendor. This product has a labeled activity of 10,000 units per mg of solid material. Enzymatic activity assay protocol from Sigma Aldrich was followed (3.4.23.1, https://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-pepsin.html). The SGF plus pepsin was freshly prepared and stored at 4°C before use. The procedure was performed as follows:

4.3.1 A solution of 25% bovine serum albumin was prepared by 25 mg/mL BSA in SGF.

4.3.2 1 mL of BSA solution (2%) was aliquoted into 4 tubes to achieve final concentrations of 2 tubes at 20 mg/mL and 2 tubes at 40 mg/mL.

4.3.3 350 µL of pepsin quenching solution (carbonate buffer) and 70 µL 5x LDS reducing buffer were added to each tube described in **4.6.1**.

4.3.4 Digestion and control samples were prepared:

4.3.4.1 200 µL pepsin stock solution is added to each solution in 4.3.2. The mixture was immediately placed in a preheated incubator (37°C) and agitated at 120 RPM to start the digestion of test sample. 200 µL aliquots from this sample were added to tubes.

4.3.4.2 200 mL pepsin stock was added to 1 mL SGF. The mixture was immediately placed in a preheated incubator (37°C) and agitated at 120 RPM to start the digestion of test sample. 200 µL aliquots from this sample were added to tubes labeled P0 and P30 at t=0 and t=30 min., respectively.

4.3.4.3 The absorbance at 280 nm was measured on a spectrophotometer (Spectramax-100). The activity units of pepsin per mL were calculated as the mean net absorbance (A280 nm BSA – 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 (BSA).

Bovine serum albumin (BSA) digestion assays were tested as control proteins to verify the appropriate activity of the test system.

4.5 Protein Digestion

Silk fibroin protein powder was stored at 4°C until immediately before use in the following digestion assay.

4.5.1 Sample Tube Preparation: 1.5 mL centrifuge tubes were labeled at P0, P60, D0, D2, D5, D10, D20, D30, D60, F0, F60.

4.5.2 70 µL of pepsin quenching solution (carbonate buffer) and 70 µl 5x LDS reducing buffer were added to each tube described in **4.6.1**.

4.5.3 Digestion and control samples were prepared:

4.5.3.1 1.43 mL (equivalent to 60 mg) fibroin stock solution was added to 570 µL pepsin stock solution. The mixture was immediately placed in a preheated incubator (37°C) and agitated at 120 RPM to start the digestion of test sample. 200 µL aliquots from this sample will be added to tubes labeled D0, D2, D5, D10, D20, D30, D60 (e.g. D2 at 2 min., D30 at 30 min).

4.5.3.2 1 mL pepsin stock was added to 1 mL SGF. The mixture was immediately placed in a preheated incubator (37°C) and agitated at 120 RPM to start the digestion of test sample. 200 µL aliquots from this sample will be added to tubes labeled P0 and P60 at t=0 and t=60 min., respectively.

4.5.3.3 1.43 mL (equivalent to 60 mg) fibroin stock solution is added to 570 µL pepsin stock solution. The mixture was immediately placed in a preheated incubator (37°C) and agitated at 120 RPM to start the digestion of test sample. 200 µL aliquots from this sample will be added to tubes labeled F0 and F60 at t=0 and t=60 min., respectively.

4.5.4. Upon addition of the sample aliquots to the quenching solution, samples were vortexed and heated in a water bath to 95°C for 5 min. in order to halt digestion. Samples were allowed to cool to room temperature before running on SDS-PAGE at 350 µg/well.

4.6 SDS-PAGE Gel

All samples on any one gel were from a single digestion experiment. NuPAGE 3-8% Tris-Acetate Protein gels were used with NuPAGE MOPS SDS Running Buffer.

4.6.1 20 µL of each sample was loaded per well, with a final protein load of 350 µg/well.

4.6.2 5 µL of PageRuler Prestained NIR Protein Ladder is loaded to the outermost wells

4.6.3 Electrophoresis was accomplished at a constant 120 V for 1.5 hrs.

4.6.4 Gels were stained for a minimum of 2 hours in InstantBlue (Coomassie), as detailed by the supplier, Expedeon.

4.7 Image Analysis

The de-stained gels were visualized by placing the gel on a lightbox (Porta-Trace, Cat.# LYSB00U6KPXAG) and capturing an image with a Canon PowerShot SX540 camera. The raw image was saved as an archival file.

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 stepwise reduced intensity of stained bands with each step in the dilution series. The minimum amount of protein that was detectable was 10 µg, equivalent to roughly 3% of the highest concentration (350 µg).

5.2 Pepsin Activity

The certified activity of the lot of pepsin from VWR International used in this study was labeled as 10,000 units per mg solid.

5.3 Control Substance Digestion Results

Stained gels of digestion tests of BSA (Figure 2) demonstrated that at both ratio of 10 units and 1 unit of pepsin activity per activity per 1 µg of test protein, BSA was digested rapidly within the SGF plus pepsin test system with more than 10% visually stainable full-length protein band remaining at 30 minutes. These results with 1 and 10 units with 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 Silk Fibroin Protein Digestion Results

Digestion of the test protein, silk fibroin, was conducted at pH 2 at the ratio of 10 units pepsin per 1 µg test protein (Figure 3). A stained gel of this digestion experiment demonstrated that silk fibroin was stable in acid alone but was rapidly digested by pepsin in under 2 minutes (Lane B) to below the detectable band intensity of the 3% undigested protein from the LOD assay (Figure 1, Lane E).

6. Conclusions

The results of this study demonstrated that the silk fibroin test protein was rapidly digested after incubation in SGF plus pepsin at 37°C at a ratio of 10 units pepsin per 1 µg test protein within 2 minutes based on InstantBlue Coomassie staining detection.

7. References

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8. Figures

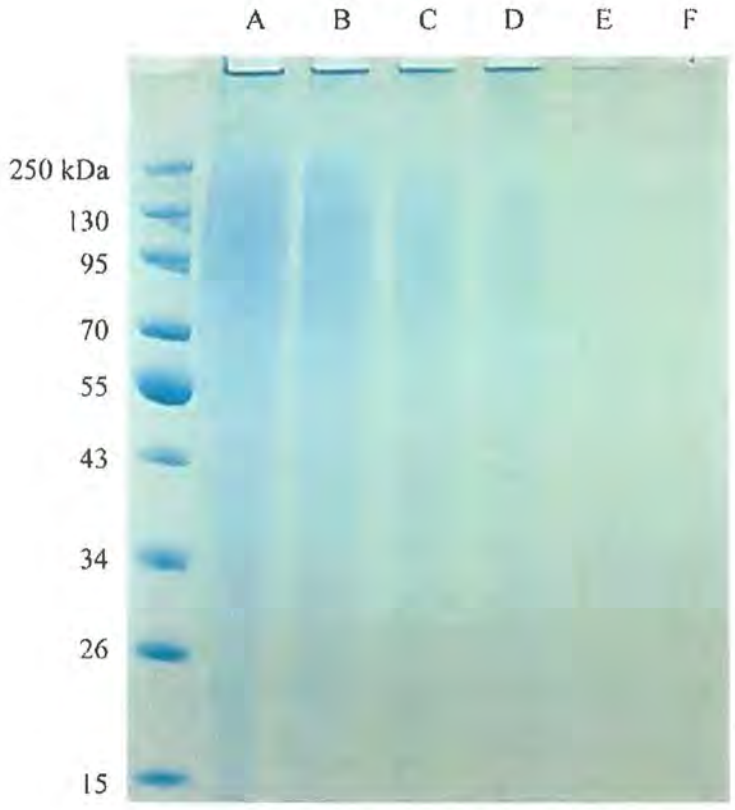

Figure 1

InstantBlue Coomassie stained SDS-PAGE gel showing serial dilution of silk fibroin starting from 100% total protein used in digestion.

Lane	Description	Protein Content
A	100% total protein	350 µg silk fibroin
B	57% total protein	200 µg silk fibroin
C	29% total protein	100 µg silk fibroin
D	14% total protein	50 µg silk fibroin
E	3% total protein	10 µg silk fibroin
F	1% total protein	3.5 µg fibroin

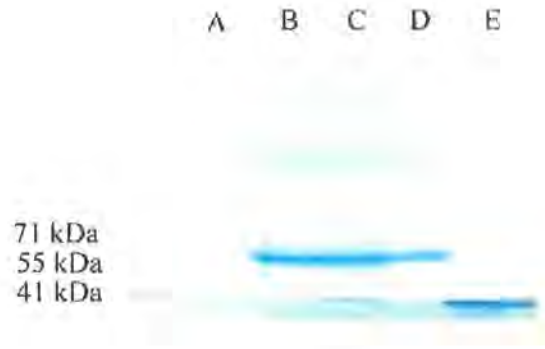

Figure 2

InstantBlue Coomassie stained SDS-PAGE gel showing the digestion of bovine serum albumin (BSA) in simulated gastric fluid at the ratio of 10 units per µg protein (pH 2.0) to measure pepsin activity. BSA was loaded at 1.47 µg per lane.

Lane	Description	Incubation time
A	Experimental control (BSA 1 mg/ml)	0 min
B	BSA in SGF (2%)	0 min
C	BSA+pepsin quenched	0 min
D	BSA (1%)+pepsin (quenched)	0 min
E	BSA + pepsin	30 min

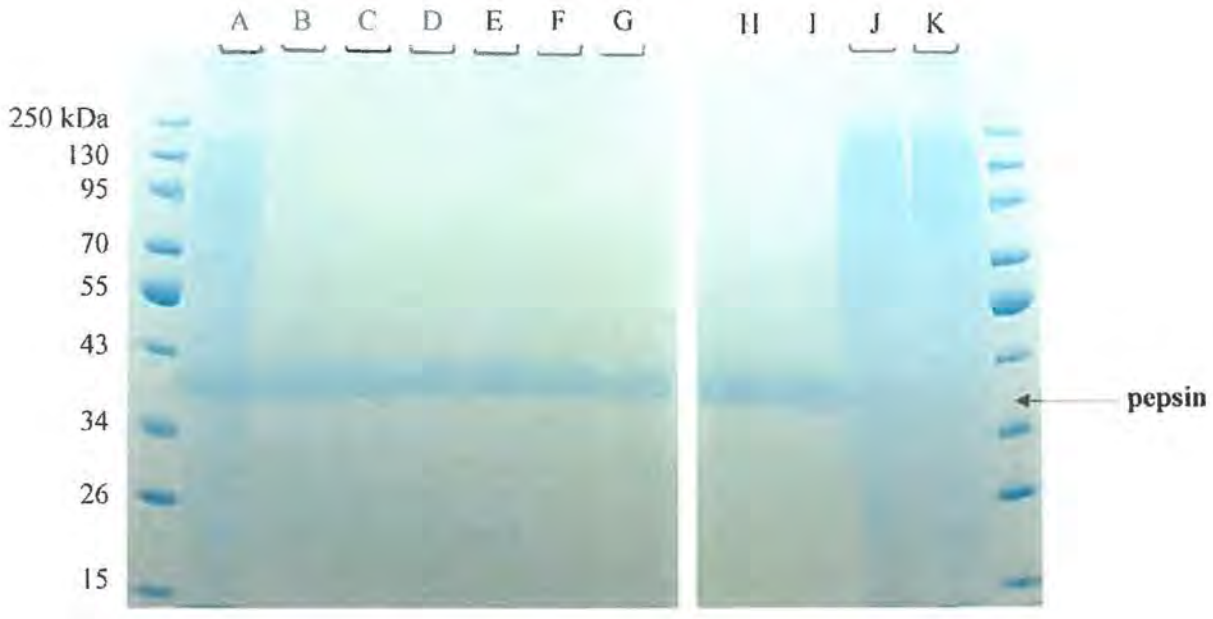

Figure 3

InstantBlue Coomassie stained SDS-PAGE gel showing the digestion of silk fibroin in simulated gastric fluid at the ratio of 10 units per 1 µg protein (pH 2.0). Silk fibroin was loaded at 350 µg per lane.

Lane	Description	Incubation time
A	Silk fibroin plus pepsin in SGF	0 min.
B	Silk fibroin plus pepsin in SGF	2 min.
C	Silk fibroin plus pepsin in SGF	5 min.
D	silk fibroin plus pepsin in SGF	10 min.
E	Silk fibroin plus pepsin in SGF	20 min.
F	Silk Fibroin plus pepsin in SGF	30 min.
G	Silk fibroin plus pepsin in SGF	60 min.
H	Control: Pepsin in SGF	0 min.
I	Control: Pepsin in SGF	60 min.
J	Control: Silk fibroin in SGF	0 min.
K	Control: Silk fibroin in SGF	60 min.

STUDY TITLE

Silk Fibroin: *in vitro* digestibility study in human simulated gastric fluid (pH 2.0) at 1 unit per µg

AUTHORS

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STUDY COMPLETED ON

6 October 2020

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LABORATORY STUDY ID

20201006 – REG – Silk Fibroin Digestion

Statement of No Data Confidentiality

No claim of confidentiality is made for any information contained in this document. I acknowledge that information not designated as within the scope of FIFRA Section 10(d)(l)(A), (B), or (C) and which pertains to a registered pesticide is not entitled to confidential treatment and may be released to the public, subject to the provisions regarding disclosure to multinational entities under FIFRA section 10(g).

Company Cambridge Crops, Inc. (d/b/a Mori)

Company Agent:

[redacted]

Laith M. Abu-Taleb, Esq.
Mori

11/12/2020
Date

These Data May Be Considered Confidential In Countries Outside The United States.

GLP 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. However, raw data and bioinformatics comparisons were archived in PDF format and remain at Mori.

Applicant/Sponsor

Sezin Yigit, Ph.D.
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Title: General Counsel, Mori

Date: 11/12/2020

Expert Review Statement

I assisted the Mori team with the design of the study outlined in this report. Together, we worked on developing the protocols based off of the Ofori-Anti *et al.* (2008) publication. I did not conduct the study in my laboratory. I did observe the results and was given a chance to view the SDS-PAGE gel as shown in the Figures in Section 8. I have reviewed this paper and agree with the conclusions made by the Mori team.

Advisor: _____

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Date: 6 October 2020

Study Number: 20200212 – REG – Silk Fibroin Digestion

Title: Silk Fibroin: *in vitro* digestibility study in human simulated gastric fluid (pH 2.0) at a ratio of 1 unit per µg

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Records Retention: All study specific raw data and a copy of the final report will be retained at Mori

Signature of Final Report Approval:

[redacted]

Principal Investigator: Sezin Yigit, Ph.D.

11/12/2020
Date

SUMMARY

The silk fibroin protein used in this study was produced and supplied by Cambridge Crops, Inc. (d/b/a Mori) of Boston, MA. The protein is derived from silkworm (*Bombyx mori*) cocoons and is comprised of three chains: the light chain, heavy chain (UniProt P05790), and glycoprotein P25. The silk fibroin test material used in this study was provided in its solid, powdered form.

The silk fibroin was subjected to digestion based on the protocol in Ofori-Anti *et al.* (2008)[1] 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 compared a non-digested sample. A limit of detection study was performed prior to digestion to ensure that 10% residual protein was detectable using SDS-PAGE and Coomassie blue staining. Due to the methods used during processing, the silk fibroin used in these studies appears as smear on SDS-PAGE. Pepsin was diluted in simulated gastric fluid (SGF) with an adjusted pH to 2.0. The pepsin solution was tested for proteolytic activity by digestion of silk fibroin within 24 hours of each assay day. The mass ratio of pepsin to silk fibroin preparation was adjusted to achieve 1 unit of pepsin activity per microgram of total protein in solution. Digestions were performed at 37°C under timed conditions. Samples of the digestion mixtures were removed and neutralized at various time points from 2 minutes 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 silk fibroin protein is rapidly digested in pepsin at pH 2.0 at a ratio of 1 activity units pepsin per microgram test protein. The SDS-PAGE Coomassie blue gel staining method demonstrated that over 75% of the silk fibroin protein was digested in less than five minutes and over 93% was digested after 60 minutes.

No degradation bands were found to result from digestion of the silk fibroin. Therefore, our conclusion is that silk fibroin is rapidly digested at a ratio of 1 unit pepsin per microgram silk fibroin in 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 silk fibroin in pepsin.

[1] Ofori-Anti, A.O., Ariyarathna, H., Chen, L., Lee, H.L., Pramod, S.N., Goodman, R.E. (2008). Establishing objective detection limits for the pepsin digestion assay used in the assessment of genetically modified foods. *Reg. Toxicol. Pharmacol.* **52**:94-103.

TABLE OF CONTENTS

1. Introduction

Mori of Boston, MA has developed a shelf-life extension technology using silk fibroin derived from the cocoons of *Bombyx mori* as the primary ingredient. Mori performed tests to determine whether there is a risk of food allergy associated with the consumption of the protein. This report describes the rationale, test methods, and results pertaining to an *in vitro* digestion assay intended to provide data indicative of potential risks of food safety.

The *Codex Alimentarius* Commission guidelines for assessing allergenicity (2003) recommends assessing the introduced protein for stability in pepsin at acidic pH in order to evaluate whether it is likely to either increase the rate of sensitization or increase the likelihood of eliciting an allergic response in consumers. The pepsin stability assay is one study in a weight of evidence approach intended to assess the potential allergenicity (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 several 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 1 unit of pepsin activity per microgram of test protein. The pure porcine pepsin protein used in this assay was purchased from VWR International, product 10791-836 (Lot # 18J2056015).

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 LDS 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 serum albumin (BSA), as described by VWR, to ensure that it is within a tolerance interval reported by VWR for that lot of enzyme. The results of our activity assay fell within the acceptance criterion of the VWR certified activity at 10,000 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 (silk fibroin 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 this, a serial dilution of test protein is run on the same SDS-PAGE and Coomassie blue staining system as the digested samples and are analyzed to evaluate a limit of detection (LOD). The LOD must be lower than 10% to perform the digestion assay. Details and results of the study are reported here.

2. Materials

2.1 Test Substance

The test substance for this study was silk fibroin isolated from the cocoons of the silkworm, *Bombyx mori*. The sample was manufactured by Mori production run 17-0202-042-P78.

2.2 Control Substance

The control substance for this study was bovine serum albumin (BSA), purchased from VWR International (Catalog # 97062-508). BSA was used to confirm the pepsin activity indicated by the manufacturer (VWR International, Catalog # 10791-836).

2.3 Reference Substance

There was no reference substance used 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 Critical Analytical Reagents

- Pepsin (VWR International, Cat.# 10791-836, Lot# 18J2056015)
- SGF without pepsin: 35 mM HCl, 123 mM NaCl (adjusted to pH = 2)
- SGF with pepsin: 10.5 mg/mL pepsin dissolved in SGF (1.05×10^5 U/mL)
- Bovine serum albumin (BSA)
- Pepsin quenching solution: 0.7 M Na_2CO_3 (pH = 11)
- NuPAGE LDS Sample Buffer (4x) (Invitrogen, Cat.# NP0007, Lot# 2020067)
- SeeBlue Pre-Stained Protein Standard (ThermoFisher, Cat.# LC5625)
- NuPAGE 4-12% Bis-Tris Protein Gels (ThermoFisher Cat.# NP0321PK2)
- NuPage MES Running Buffer (20x) (ThermoFisher Cat.# NP000202)
- InstantBlue Protein Stain (Expedeon, Cat.# ISB1L)

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 Sigma Aldrich for determining the activity of pepsin. Pepsin powder was dissolved in prepared SGF, pH 2.0, to provide a final concentration of 2 mg/ml. Acidified bovine serum albumin (2% mass to volume) was prepared and digested in triplicate to evaluate the labeled pepsin activity.

The amount of pepsin powder used to prepare SGF was calculated from the specific activity labeled on the product as 10,000 units/mg solid pepsin product. 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 1 unit of pepsin activity per microgram test protein. Pepsin stock is dissolved in SGF at a concentration of 10.5 mg/mL (1.05×10^5 U/mL). In addition, a silk fibroin stock was made at 42 mg/mL in SGF. The digestion reaction mixture was made by mixing 1.43 mL silk fibroin stock solution and 570 µL pepsin stock solution. This achieves the desired ratio of 1 unit pepsin per microgram silk fibroin protein in 2 mL. The reaction mixture is placed in a preheated incubator at 37 °C and gently agitated at 120 RPM for the duration of the experiment.

Once the test protein solution was placed in the incubator (37 °C), 200 µL aliquots were withdrawn at predetermined times (t = 0, 2, 5, 10, 20, 30, 60 min.) and added to test tubes containing a mixture of 70 µL 0.7 M Na_2CO_3 (pH = 11) and 70 µL 5x LDS buffer, for a total of 340 µL. Upon addition of the test solution to the denaturing reagents (LDS buffer and Na_2CO_3), the samples are immediately heated in a water bath to 95 °C for 5 min. in order to halt digestion. Samples were allowed to cool to room temperature before running on SDS-PAGE at 350 µg/well.

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 (t = 0 and 60 min.), and pepsin alone (t = 0 and 60 min.) to asses pepsin stability throughout the duration of the assay.

Samples were separated by electrophoresis, stained with InstantBlue Protein Stain, and images were captured using a Canon PowerShot SX540 camera. 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 well in the LOD assay. 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 commonly used 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. 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 measures the resistance of a test protein to proteolysis in a test tube system. It is not meant to be a stand-alone determinant in evaluating the potential allergenicity of proteins 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 via SDS-PAGE
- Inclusion of samples of pepsin without test protein at t = 0 and 60 min. 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.

4. Detailed Study Methods

This study evaluated the stability of silk fibroin, derived from the cocoons of *Bombyx mori*, in pepsin in SGF at pH = 2. Several control steps were performed to ensure study validity. A detailed description of the study is presented here. Laboratory records and protocols are on file at Mori in Boston, MA and may be made available upon request.

4.1 Verification of Detection System and Sensitivity

A dilution series of test sample was prepared with sample quantities loaded in SDS-PAGE gel using 4x LDS buffer, covering a range from 100% total protein per well (350 µg) to <1% total protein per well (2.7 µg). Following electrophoresis, the gels were stained with InstantBlue (Coomassie) for at least 2 hours. The gels were washed with deionized water three times until the background was clear. The image was captured using Canon PowerShot SX540 camera.

4.2 Preparation of SGF Plus Pepsin

The simulated gastric fluid (SGF) was prepared by mixing 1.75 mL 1.0 M HCL with 48.25 mL diH_2O and then adding 359.4 mg NaCl. This achieves a final SGF solution concentration of 35 mM HCl and 123 mM NaCl. The activity of pepsin purchased from VWR International was verified to be 10,000 U/mg. A stock pepsin solution was prepared by adding 105 mg pepsin to 10 mL SGF. This achieves a final pepsin stock concentration of 10.5 mg/mL (1.05×10^5 U/mL). 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

The activity of pepsin was verified with Bovine Serum Albumin ("BSA") to ensure the activity units claimed by the vendor. This product has a labeled activity of 10,000 units per mg of solid material. Enzymatic activity assay protocol from Sigma Aldrich was followed (3.4.23.1, https://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-pepsin.html). The SGF plus pepsin was freshly prepared and stored at 4℃ before use. The procedure was performed as follows:

4.3.1 A solution of 25% bovine serum albumin was prepared by 25 mg/mL BSA in SGF.

4.3.2 1 mL of BSA solution (2%) was aliquoted into 4 tubes to achieve final concentrations of 2 tubes at 20 mg/mL and 2 tubes at 40 mg/mL.

4.3.3 350 µL of pepsin quenching solution (carbonate buffer) and 70 µL 5x LDS reducing buffer were added to each tube described in **4.6.1**.

4.3.4 Digestion and control samples were prepared:

4.3.4.1 200 µL pepsin stock solution is added to each solution in 4.3.2. The mixture was immediately placed in a preheated incubator (37° C) and agitated at 120 RPM to start the digestion of test sample. 200 µL aliquots from this sample were added to tubes.

4.3.4.2 200 mL pepsin stock was added to 1 mL SGF. The mixture was immediately placed in a preheated incubator (37°C) and agitated at 120 RPM to start the digestion of test sample. 200 µL aliquots from this sample were added to tubes labeled P0 and P30 at t=0 and t=30 min., respectively.

4.3.4.3 The absorbance at 280 nm was measured on a spectrophotometer (Spectramax-100). The activity units of pepsin per mL were calculated as the mean net absorbance (A280 nm BSA – 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 (BSA).

Bovine serum albumin (BSA) digestion assays were tested as control proteins to verify the appropriate activity of the test system.

4.5 Protein Digestion

Silk fibroin protein powder was stored at 4° C until immediately before use in the following digestion assay.

4.5.1 Sample Tube Preparation: 1.5 mL centrifuge tubes were labeled at P0, P60, D0, D2, D5, D10, D20, D30, D60, F0, F60.

4.5.2 70 µL of pepsin quenching solution (carbonate buffer) and 70 µL 5x LDS reducing buffer were added to each tube described in **4.6.1**.

4.5.3 Digestion and control samples were prepared:

4.5.3.1 1.43 mL (equivalent to 60 mg) fibroin stock solution was added to 570 µL pepsin stock solution. The mixture was immediately placed in a preheated incubator (37°C) and agitated at 120 RPM to start the digestion of test sample. 200 µL aliquots from this sample will be added to tubes labeled D0, D2, D5, D10, D20, D30, D60 (e.g. D2 at 2 min., D30 at 30 min).

4.5.3.2 1 mL pepsin stock was added to 1 mL SGF. The mixture was immediately placed in a preheated incubator (37°C) and agitated at 120 RPM to start the digestion of test sample. 200 µL aliquots from this sample will be added to tubes labeled P0 and P60 at t=0 and t=60 min., respectively.

4.5.3.3 1.43 mL (equivalent to 60 mg) fibroin stock solution is added to 570 µL pepsin stock solution. The mixture was immediately placed in a preheated incubator (37°C) and agitated at 120 RPM to start the digestion of test sample. 200 µL aliquots from this sample will be added to tubes labeled F0 and F60 at t=0 and t=60 min., respectively.

4.5.4. Upon addition of the sample aliquots to the quenching solution, samples were vortexed and heated in a water bath to 95°C for 5 min. in order to halt digestion. Samples were allowed to cool to room temperature before running on SDS-PAGE at 350 µg/well.

4.6 SDS-PAGE Gel

All samples on any one gel were from a single digestion experiment. NuPAGE 4-12% Bis-Tris Protein gels were used with NuPAGE MES Running Buffer.

4.6.1 20 µL of each sample was loaded per well, with a final protein load of 350 µg/well.

4.6.2 5 µL NuPage SeeBlue Protein Standard is loaded to the outermost wells

4.6.3 Electrophoresis was accomplished at a constant 120 V for 1.5 hrs.

4.6.4 Gels were stained for a minimum of 2 hours in InstantBlue (Coomassie), as detailed by the supplier, Expedeon.

4.7 Image Analysis

The de-stained gels were visualized by placing the gel on a lightbox (Porta-Trace, Cat.# LYSB00U6KPXAG) and capturing an image with a Canon PowerShot SX540 camera. The raw image was saved as an archival file.

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 stepwise reduced intensity of stained bands with each step in the dilution series. The minimum amount of protein that was detectable was 5.5 µg, equivalent to roughly 1.6% of the highest concentration (350 µg).

5.2 Pepsin Activity

The certified activity of the lot of pepsin from VWR International used in this study was labeled as 10,000 units per mg solid.

5.3 Control Substance Digestion Results

A stained gel of a digestion test of BSA (Figure 2) demonstrated that at ratio of 1 unit of pepsin activity per activity per 1 µg of test protein, BSA was digested rapidly digested in under 2 minutes. These results 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 Silk Fibroin Protein Digestion Results

Digestion of the test protein, silk fibroin, was conducted at pH 2 at the ratio of 1 unit pepsin per 1 µg test protein (Figure 3A). A stained gel of this digestion experiment demonstrated that silk fibroin was stable in acid alone but was rapidly digested by pepsin at a ratio of 1 activity unit per microgram. In particular, over 75% of the silk fibroin was digested in less than five minutes (Figure 3A, Lane C) and over 93% was digested within 60 minutes (Figure 3A, Lane G).

6. Conclusions

The results of this study demonstrated that the silk fibroin test protein was significantly digested (over 75%) after incubation in SGF plus pepsin at 37°C at a ratio of 1 unit pepsin per 1 µg test protein within 5 minutes based on InstantBlue Coomassie staining detection. Within 60 minutes, over 93% was digested.

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8. Figures

1.

Lane	Description	Protein Content
A	100% total protein	350 µg silk fibroin
B	50% total protein	175 µg silk fibroin
C	25% total protein	88 µg silk fibroin
D	12.5% total protein	44 µg silk fibroin
E	6.3% total protein	22 µg silk fibroin
F	3.1% total protein	11 µg fibroin
G	1.6% total protein	5.5 µg fibroin
H	0.8% total protein	2.7 µg fibroin

Figure 1

InstantBlue Coomassie stained SDS-PAGE gel showing serial dilution of silk fibroin starting from 100% total protein used in digestion.

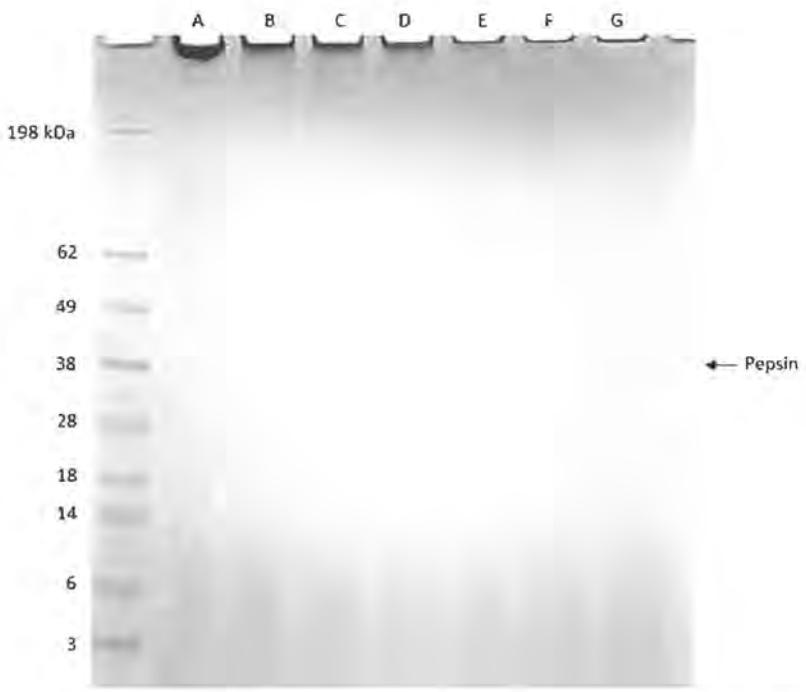
2.

Lane	Description	Incubation time
A	BSA plus pepsin in SGF	0 min.
B	BSA plus pepsin in SGF	2 min.
C	BSA plus pepsin in SGF	5 min.
D	BSA plus pepsin in SGF	10 min.
E	BSA plus pepsin in SGF	20 min.
F	BSA plus pepsin in SGF	30 min.
G	BSA plus pepsin in SGF	60 min.

Figure 2

InstantBlue Coomassie stained SDS-PAGE gel showing digestion of BSA by pepsin in SGF at a ratio of 1 unit activity per µg protein. Samples taken at 0, 2, 5, 10, 20, 30, and 60 minutes in lanes A-G, respectively.

3A.



Lane	Description	Incubation time
A	Silk fibroin plus pepsin in SGF	0 min.
B	Silk fibroin plus pepsin in SGF	2 min.
C	Silk fibroin plus pepsin in SGF	5 min.
D	silk fibroin plus pepsin in SGF	10 min.
E	Silk fibroin plus pepsin in SGF	20 min.
F	Silk Fibroin plus pepsin in SGF	30 min.
G	Silk fibroin plus pepsin in SGF	60 min.

3B.

Lane	Description	Incubation time
A	Pepsin in SGF	0 min.
B	Pepsin in SGF	60 min.
C	BSA in SGF	0 min.
D	BSA in SGF	60 min.
E	Silk fibroin in SGF	0 min.
F	Silk Fibroin in SGF	60 min.

Figure 3

(A) InstantBlue Coomassie stained SDS-PAGE gel showing digestion of silk fibroin by pepsin in SGF at a ratio of 1 unit activity per µg protein. Samples taken at 0, 2, 5, 10, 20, 30, and 60 minutes in lanes A-G, respectively. (B) Stability of test system proteins in SGF at 0 and 60 minutes.

APPENDIX J
SUPPLEMENTARY PUBLIC MATERIALS

APPENDIX J1
PEER REVIEWED PUBLICATION

Sixteen pages have been removed in accordance with copyright laws. The removed reference citation is:

Sezin Yigit, “Toxicological assessment and food allergy of silk fibroin derived from Bombyx mori cocoons”, Food and Chemical Toxicology 151 (2021) 112117

APPENDIX J2
Peer-reviewed abstract and poster for Food Anaphylaxis Meeting as sponsored by the European Academy of Allergy & Clinical Immunology (EEACI), October 16, 2020

POSTER ABSTRACT SESSION 9

Food allergy safety assessment of extracted silk proteins used in food protection by bioinformatics, mass spectrometry and digestion in pepsin

Richard E Goodman

Abstract

FP95

Food allergy safety assessment of extracted silk proteins used in food protection by bioinformatics mass spectrometry and digestion in pepsin

Richard E Goodman1, Philip E Johnson1, Sezin Yigit2, James L. Sugarman2, Laith Abu-Taleb2, Adam Behrens2

1University Of Nebraska, 2Mori

INTRODUCTION: Unique properties of various silk proteins is leading to investigations and use in food, medicinal, and cosmetic applications. When used in food, target proteins are extracted by first boiling cocoons in a high pH solution and then washing in a saline solution. The salt is removed via dialysis, leaving desired proteins in solution. This Silk Protein Solution (SPS) is applied to foods and it forms an edible film encapsulating the food and extending its shelf-life by reducing oxidation and improving moisture retention. The potential for widespread use and consumption necessitates an evaluation of potential risks of food allergy.

METHODS: The allergenic potential was assessed using methods recommended in the CODEX Alimentarius guideline (2003) for food proteins, with the addition of liquid chromatography-mass spectrometry (LCMS) for detection of allergenic insect proteins. Food allergy to consumption of silkworm pupae has been described and is likely due to five known allergenic proteins. Digestion of silk proteins by pepsin was tested. The amino acid sequences of protein components of silk fibroins were subjected to bioinformatic analyses compared to allergens in www.AllergenOnline.org using full-length FASTA3 a sliding 80 AA sequence identity matches >35% or 8 AA identity matches to any allergen. The sequences were also compared to and NCBI (Entrez Protein) using BLASTP.

RESULTS: The five allergenic proteins were readily detected in silkworm pupae but were not found in the cocoon before or after processing, nor in the final SPS product. Proteins in the SPS were readily digested in 10 units of pepsin per microgram SPS. Bioinformatics searches of the fibroin proteins showed no matches of >50% identity that would suggest likely cross-reactivity. Sixteen matches were found to fibroin heavy chain precursor >35% and less than 45% identity over 80 AA using FASTA. However, these were low intensity matches and are unlikely to represent risks of allergic cross-reactivity.

CONCLUSION: Based on the protein sequence comparisons, LCMS, and digestion studies, it was determined that SPS does not contain known allergens, or potentially IgE-cross-reactive proteins based on the best-practice allergenicity tools employed.

Food allergy safety assessment of extracted silk proteins used in food protection: bioinformatics, mass spectrometry and digestion in pepsin

Richard E. Goodman*[1], Philip E. Johnson[1], Sezin Yigit[2], James L. Sugarman[2], Laith M. Abu Taleb[2], Adam M. Behrens[2]

[1]University of Nebraska, Lincoln, Nebraska, United States. *corresponding author rgoodman2@unl.edu, [2] Mori, formerly Cambridge Crops, Boston, MA

INTRODUCTION

- Cambridge Crops (now Mori) developed technology to extend the shelf-life of foods, including whole- and cut-produce, processed foods, meats, and fish. A solution of silk proteins from *Bombyx mori* cocoons, primarily fibroin, are extracted via salt and water. The proteins have been tested for safety in toxicology studies (www.bit.ly/CambridgeCropsSafety).
- The product evaluated for potential food allergy risks to ensure consumer safety
- Evaluation followed CODEX guidelines for GE crops
  - Characterization of proteins in the product including amino acid sequences
  - Bioinformatics searches by AllergenOnline.org and NCBI Protein database for matches to allergens
  - Test the stability of proteins in pepsin at pH 2

AIMS

- Identify proteins in silk extract using DDA MS
- Select possible allergenic proteins from literature searches for allergy to silk cocoons and pupae
- Determine whether identified proteins in the product have sequence identities similar to known allergens
- Evaluate silk protein residues following digestion in pepsin

1. PRODUCTION of Fibroin

- *Bombyx mori* cocoons
- Cut, removed pupae
- Boiled empty cocoons at high pH to removed sericin
- Immobile fibroin rinsed in H_2O
- Solubilized in saline
- Dialyzed to remove salt
- Soluble fibroin protein powder to coat foods

2. Literature Search allergy & allergens

- PubMed
- Google
- Downloaded & read papers
- Searched in WHO/IUIS Allergen.org
- Searched AllergenOnline.org

3. Stability in Pepsin

- Silk fibroin total protein measured, in dH_2O
- Digested with 10 units pepsin (VWR #10791-836 per ug protein or with 1 unit per ug protein with shaking at 37°C
- BSA digested as a control
- Timed sample stopped with Na-Carbonate and 5X Laemmli buffer with heat
- Samples on SDS-PAGE reducing gels and stained with Coomassie blue

4. Mass Spectrometry (Protein ID)

- Pupa, cocoon and two silk fibroin solutions were reduced & alkylated, then digested with trypsin
- LC-MS analysis was performed using Orbitrap Elite Hybrid Ion Trap-Orbitrap MS
- Data analysis with PEAKSQ version 8.5 with database of Bombyx mori proteins from UniPro, reporting with Filter aided sample prep, DDA MS

5. Bioinformatics AllergenOnline.org and BLASTP vs Protein

- The amino acid sequences ofpProteins of fiboins that were identified from extracts of silk were compared to known allegens using
  - Full FASTA
  - Sliding 80mer FASTA
  - And 8 AA identity matches
- Identified proteins were also compared to the NCBI protein database using BLASTP

6. RESULTS Literature Jan '20

- 6 papers minor suggestion that samples with sericin MIGHT be an allergen
- A few pupal proteins are likely allergens
- No fibroin proteins are described as allergenic

7. DDA Mass Spectrometry protein identification

DDA MS Scores (MUPO Quality) highest -10 log P

Putative Allergen / Samples	Tropomyosin	Arginine kinase	Thioredoxin	Chitinase	Paramyosin
Pupal	237.67 (7)	313.42(10)	227.14(5)	348.47(13)	159.65(5)
Cocoon	nd (0)	nd (0)	nd (0)	nd (0)	nd (0)
Degummed fibroin	nd (0)	nd (0)	nd (0)	nd (0)	nd (0)
Fibroin Powder	nd (0)	nd (0)	nd (0)	nd (0)	nd (0)

Bioinformatics Results: AllergenOnline.org version 20

Protein	Full Fasta AOL	Sliding 80mer Best score	8mer
Fibroin heavy (5263 AA)	Weak, 26% ID E score 2e-26 Collagen cow	45% ragweed pollen allergen	NA
Fibroin light (262 AA)	Weak 30% ID E score 0.14 Globin insect	NA	NA
Fibroin P25 (220 AA)	Weak 26% ID E score 0.89 Fire ant PLA	NA	NA
Sericin (622 AA)	Weak 28% ID E score 6e-10 Chicken vitellogenin	46% chicken vitellogenin	NA
Ber e 1 2S albumin (146 AA) Allergen control	STRONG 100% ID E score 4e-31 Brazil nut 2S	100% Ber e 1	YES many

CONCLUSIONS: Potential risk of food allergy? VERY LOW, should not be a problem

001117

APPENDIX J3
Materials Published to the International Association for Food Protection Annual Event, October 28, 2020

Twenty-two pages have been removed in accordance with copyright laws. The removed reference citation is:

Sezin Yigit, “Evaluating the Safety and Potential Risks of Food Allergy of Silk Fibroin Derived from Bombyx mori Cocoons” Preprints (www.preprints.org) I NOT PEER-REVIEWED I Posted: 23 October 2020

APPENDIX K
HEALTH CANADA LETTER

Health Canada / Santé Canada
Health Products and Food Branch / Direction générale des produits de santé et des aliments

Bureau of Microbial Hazards
251 Sir Frederick Banting Driveway
Mail Stop #2204E
Ottawa, Ontario
K1A 0K9

June 21, 2021

Laith Abu-Taleb
Cambridge Crops, Inc.
440 Rutherford Ave
Boston, MA, United States of America
02129

Subject: Non-Novelty Determinations Regarding Mori Silk™ (Case # 2021-025133)

Dear Mr. Abu-Taleb:

This letter refers to Cambridge Crops Inc's novelty determination concerning the food use of Mori Silk™ for human consumption. Officers of the Food Directorate, Health Products and Food Branch, have reviewed the information provided by you to determine the potential novelty of these foods ad defined in *Division 28 of the Food and Drug Regulations*.

Briefly, Mori Silk™ is a fibroin protein isolate from silkworm (*Bombyx mori*) cocoons. The protein isolation process involves treating silkworm cocoons with salt, water and heat. As established by the supplied references, silkworms, silkworm cocoons as well as silkworm cocoon protein isolate all have a history of food use. In addition, the described protein isolation process was not considered to be a novel process.

Based on the information provided, we have concluded that Mori Silk™ fibroin protein isolate has a history of safe use as a food and is not subject to a pre-market notification under B.28.002 of the *Food and Drug Regulations*. It should be noted that this opinion is only in regard to the novelty status of this product and does not constitute a safety assessment or a confirmation that the ingredient is compliant in all aspects with the *Food and Drug Regulations*.

It is the continuing responsibility of a manufacturer or importer to ensure that their products are in compliance with all applicable statutory and regulatory requirements.

.../2

The sale of a food or food ingredient that poses a hazard to the health of consumers would contravene the provisions of the *Food and Drugs Act*.

Finally, please note that the Canadian Food Inspection Agency announced the *Safe Foods for Canadians Regulations*. If you are a food business that imports or prepares food for export or to be sent across provincial or territorial boundaries, there are new licensing, preventive control and traceability requirements that you will have to meet. We recommend that you consult the CFIA's website at: http://inspection.gc.ca/food/eng/1299092387033/1299093490225 to verify whether these new requirements apply to your business.

Sincerely yours,

Luc Bourbonnière
Chief, Evaluation Division
Bureau of Microbial Hazards
Food Directorate

GRAS Notice GRN 1026 amendments

From: Laith Abu-Taleb
To: Kampmeyer, Christopher
Subject: [EXTERNAL] Re: Regarding your submission to the FDA GRAS Notification Program
Date: Wednesday, October 6, 2021 12:41:02 PM
Attachments: U.S.%20Food%20and%20Drug%20Administration
Follow%20FDA%20on%20Facebook
Follow%20FDA%20on%20Twitter
View%20FDA%20videos%20on%20YouTube
View%20FDA%20photostream%20on%20Flickr
Subscribe%20to%20FDA%20RSS%20feeds

Dear Mr. Kampmeyer,

Thank you for your email. We confirm that:

1) the GRAS Notice for silk protein in food is submitted in accordance with 21 CFR Part 170, Subpart E per 21 CFR ~~Part~~ 170.225(c)(1);

(2) any information marked as "trade secret/confidential" is **not confidential** and may be made public; and

(3) the intended use of silk protein in fish **excludes** catfish.

We look forward to having our GRN filed and reviewed by FDA. For background, as you likely know, the Notice is a resubmission of GRN 930, which we requested FDA to Cease to Evaluate. The major impediment to GRN 930 was the lack of peer-reviewed publication at the time the notice was filed, which is now published. That, plus a more accurate description of exposure estimates as discussed with Dr. Eischeid and the prior review team, should complete the issues raised regarding this GRN.

I'd be happy to answer any additional questions you may have. Sincerely appreciate you and your team working on this.

Best regards,
Laith

On Mon, Oct 4, 2021 at 2:12 PM Kampmeyer, Christopher <Christopher.Kampmeyer@fda.hhs.gov> wrote:

> Dear Mr. Abu-Taleb:

I am writing regarding your submission dated July 7, 2021, regarding uses of “silk protein” in food to the GRAS Notification Program. During our initial review, we noted some points of clarification; could you please confirm, in response to this email:

-
-
-

Thank you,

Chris

Chris Kampmeyer, M.S.

Regulatory Review Scientist

Office of Food Additive Safety

Center for Food Safety and Applied Nutrition

U.S. Food and Drug Administration

christopher.kampmeyer@fda.hhs.gov

--

Laith Abu-Taleb, Chief Strategy Officer & General Counsel | www.mori.com | (c) +1.202.834.3174 | laith@mori.com | Bio

sender and delete it from your mailbox. Thank you.

formerly Cambridge Crops

From: Laith Abu-Taleb
To: Gaynor, Paulette M
Subject: [EXTERNAL] Re: GRN 1026 - items for clarification
Date: Wednesday, March 30, 2022 5:09:43 PM
Attachments: 2022.03.30.Re GRN 1026_Mori Silk.pdf

Dear Dr. Gaynor,

We hope you and the team are doing well.

Please find attached our answers to your questions sent to us via email. If there are any further questions, please do let me know.

Thank you very much.

Best regards,
Laith

On Thu, Mar 24, 2022 at 2:57 PM Laith Abu-Taleb <laith@mori.com> wrote:

> Dr. Gaynor,
>
> Thank you for your correspondence. We are working diligently on the answers and will do our best to respond by March 30th, 2022.
>
> Best regards,
> Laith
>
> On Wed, Mar 16, 2022 at 12:30 PM Gaynor, Paulette M <Paulette.Gaynor@fda.hhs.gov> wrote:
>
> > Laith M. Abu-Taleb, Esq.
> > Cambridge Crops, Inc. d/b/a Mori
> > *By email*: laith@mori.com
> >
> > Dear Mr. Abu-Taleb,
> >
> > As you noted in GRN 1026 (dated July 7, 2021), this notice is a resubmission of GRN 930. As

you are aware, the cover letter to GRN 1026 includes a partial email thread about GRN 930. Regarding this email thread about GRN 930, we would like to remind you that there was a subsequent email from FDA on June 15, 2021, noting that a cease to evaluate letter summarizes aspects of the notice that did not meet requirements for a GRAS conclusion, and the letter we issued is consistent with that purpose.

During our continuing evaluation of GRN 1026, our team has been made aware of a *Federal Register* document pertaining to declaration of ingredients (58 FR 2850, January 6, 1993). And, we are making you aware of this document.

As we continue with our evaluation of GRN 1026, we have identified items that require clarification. These items follow; *note pages cited below are based on the numbering system in the Table of Contents in GRN 1026*:

1. On p. 10 (Table 2), you compare the molecular weights of silk fibroin reported in the literature to the molecular weight of silk fibroin that is the subject of GRN 1026 (this notice) and state that based on the SDS-PAGE results, the peak molecular weight (MW) for this silk fibroin is approximately 460 kDa. We note that the MW of this silk fibroin is higher than the MW of the native silk fibroin (heavy chain of ~390 kD and light chain of ~25-26 kD) and the literature MWs listed in Table 2 (390 kDa and 375-446 kDa). We also note that the degumming and dissolution processes lead to different degrees of degradation of native silk fibroin, resulting in lower MWs and different MW distribution of the regenerated silk fibroin, depending on the conditions used for these processes.

    a. Please explain the presence of fragments with MWs greater than that present for the native protein and the literature MWs.

    b. Please clarify if MWs provided in the first paragraph in Part 2.1 on p. 6 are representative of silk fibroin that is the subject of this notice or native silk fibroin and state an average MW and MW range for silk fibroin that is the subject of this notice.

2. Please confirm that the manufacturing process do not result in formation of silk fibroin nanoparticles. In addition, please provide data demonstrating the particle-size distribution for silk fibroin that is the subject of this notice.

3. On p. 8 (Part 2.4), you state that only food-grade substances and processing aids are used in the manufacture of silk fibroin. Please confirm that alkaline substances used in the degumming process are authorized by a regulation or concluded to be GRAS for such use in the U.S. and that water is the only solvent used in the manufacturing process.

4. On p. 8 (Part 2.4), you state that calcium chloride or “other similar GRAS salts” are used to dissolve the degummed silk fibers. Please clarify what other salts besides calcium chloride may be used in the dissolution step.

5. On p. 8 (Part 2.4), you state that silk fibroin is easily separated from sericin by boiling cocoons in an alkaline solution and thoroughly rinsing with water. On p. 6 (Part 2.1), you note that fibroin and sericin can be separated during the degumming process, and the removal of sericin during the degumming process can be verified. Please discuss the potential for residual sericin being present in the final silk protein or describe how you ensure that it is completely removed from the final silk fibroin.

6. On p. 3 (Table 3a), EPA method 200.8 is identified as the analytical method used to analyze for heavy metals. We note that in the certificates of analysis (Appendix C2), the method was identified as TP-A055. Please clarify this discrepancy.

7. On p. 3 (Table 3a), you identify USDA/FSIS MLG 8.05 as the method used to test for *Listeria monocytogenes*. We note that this method has been revised and its current version is MLG 8.13 (https://www.fsis.usda.gov/news-events/publications/microbiology-laboratory-guidebook). Please confirm that the correct citation for the method that you use to test for *L. monocytogenes* is MLG 8.13.

8. On p. 12, you state silk fibroin consists of 98.6% protein on a dry weight basis. Please describe the composition of the remaining 1.4%.

9. On p. 13 (Table 4), the levels of arsenic and lead in each of the three silk fibroin batches are reported to be <0.5 mg/L. We note that the specification limit for each of these heavy metals is <1 mg/L. We request that you reconsider the specification limits for arsenic and lead. The specification limits should reflect the results of batch analyses and be as low as possible.

10. On p. 13 (Table 4), the results of batch analyses for *Listeria monocytogenes* and *Salmonella* serovars are reported as “Negative/10g”; however, the specification limits for these microorganisms are stated to be “Negative/25g”. Please explain the discrepancy between the sample size identified in the specification limits and in the results of the batch analyses.

11. On p. 13 (Table 4), the value reported for the carbohydrate content in Batch 342 is 0.1%. We believe that the value should be reported as <0.1%. Please confirm that it was an omission and that <0.1% is the correct value for the carbohydrate content in Batch 342.

    In addition, we note that calcium is listed in Table 4 as one of the additional parameters that is measured in silk fibroin. We note that calcium is not listed on p. 12 in Table 3b. Please clarify if calcium is one of the additional parameters that is periodically measured in silk fibroin.

12. On p. 2, you state that silk fibroin is used as a coating “to preserve food in accordance with allowed mechanisms described 21 CFR 170.3 (o)” and “intended to extend the shelf life of foods by forming a protective barrier on the outside of the food.” On p. 14,

you reiterate that silk fibroin is intended to be used as a coating "to preserve food in accordance with allowed mechanisms described in 21 CFR 170.3(o), including as a surface finishing agent." We note that according to 21 CFR 170.3(o), mechanisms to preserve food also include mechanisms that are attributed to antimicrobial agents and antioxidants.

Please clarify if the intended use of silk fibroin includes use as an antimicrobial agent and/or antioxidant. We note that if you intend to use silk fibroin as an antimicrobial agent you would need to provide data demonstrating the antimicrobial effect of silk fibroin in representative foods, including the quantity of silk fibroin required to produce such an effect.

13. In Table H1 (Appendix H), you list subcategories of foods in which you intend to use silk fibroin, including fish fillet, shellfish (assumed peeled), and seafood eaten raw. Please describe the specific technical effects of silk fibroin in these food subcategories and state if the intended use of silk fibroin in these foods will result in changes in color, odor, texture and/or flavor that could mislead consumers about the quality (freshness) of these foods or mask deterioration of these foods in a manner that might affect the safe consumption of fish and shellfish. In addition, please discuss whether you considered the safety of silk fibroin when it is used on foods that are cooked, fried, or otherwise heated during food preparation.

14. On October 6, 2021, you provided an update that the intended use in fish excludes catfish. We note that Table H5 (Appendix H) includes multiple food codes for catfish, indicating that silk fibroin is intended for use as a coating on catfish. We note that all wild-caught and farm-raised *Siluriformes* fish sold for human food are subject to regulation by the U.S. Department of Agriculture (USDA). We request that you address the following:

    c. Please clarify whether silk fibroin is intended for use in catfish. If you intend to use silk fibroin in catfish or in any other foods that are under USDA's jurisdiction, you would need to provide data demonstrating that silk fibroin is suitable for use in such foods.

    d. Please clarify whether the food codes corresponding to catfish listed in Table H5 were considered in the dietary exposure assessment. If you do not intend to use silk fibroin in catfish, please provide a revised dietary exposure assessment that excludes uses of silk fibroin in catfish.

15. We note that Table H5 (Appendix H) contains several food codes that represent foods with standards of identity (e.g., chocolate, sweet or dark; cheddar cheese). Please provide a statement that silk fibroin is not intended for use in foods where standards of identity would preclude its use.

16. On p. 17 (Table 5), you provide the intended use levels of silk fibroin in selected food categories. Please confirm that the use levels are provided based on dehydrated silk fibroin, not the silk fibroin solution.

17. On pp. 17-21 (Tables 6-7), you provide the estimates of dietary exposure to silk fibroin. Please confirm that the estimates represent the dietary exposures for the eaters-only population.

18. We note that the silk fibroin used as a test material in the toxicological studies published by Yigit et al. 2021 was manufactured using lithium bromide in the dissolution step. Please confirm that silk fibroin manufactured using calcium chloride (or other salts that you intend to use) is the same as the test material used in the toxicological studies and that this change in the manufacturing process does not affect your safety conclusion.

19. We note that the GRAS expert panel report (Appendix A) includes Tables 1 and 2 containing outdated specifications and dietary exposure estimates. Please provide a statement addressing this discrepancy with the current notice (i.e., GRN 1026) and confirm that it did not affect your safety conclusion.

20. In Part 6.4, you describe proteins of allergenic interest (tropomyosin, arginine kinase, chitinase, paramyosin) identified in the silkworm pupae, cocoons, degummed fibroin, or Mori silk. Further, you include, in Appendix F, a proteomics analysis report (unpublished from Dr. Philp Johnson, including his "expert opinion that the fibroin powder product does not contain Proteins of Allergenic Interest that are detectable using the method employed." Please provide a statement for the record that silk fibroin does not contain any of the proteins of allergenic interest and briefly discuss steps in the method of manufacture that ensure absence of allergenic protein in the silk fibroin ingredient.

21. Please address whether silk fibroin, the subject of this GRAS notice, is **produced** in adult insects; and, please cite the relevant references. You may address the question by addressing the following:

    e. Whether the silk glands producing silk fibroin are only active in larvae or are also active in adult insects.

    f. Whether the silk fibroin-encoding gene/genes are active only in larvae or are also active in adult insects.

    g. **If silk is produced in adult insects**, please address the following:

        i. Are they produced from the same silk gland that produces this protein in larvae?

        ii. Distribution of silk fibroin in adult insects, that is, whether it is found in the exoskeleton of the adult insect, or also in non-exoskeletal parts of the insect (including internally).

        iii. Identity of silk fibroin in adult insects, that is, whether the adult silk fibroin identical in sequence to the one produced in larvae, and

        iv. Relative amounts of silk fibroin (w/w of tissue) in adults compared to

that in larvae.

22. GRN 1026 includes a 'GRAS expert panel report' (Appendix A). As discussed in the GRAS final rule (81 FR 54960, August 17, 2016), convening such a panel is not required. Nevertheless, as the panel was convened on your behalf, we are seeking clarification about materials that the panel considered given the signatures on the panelists report are from April 2020.

    h. In Part 1.5, you refer to Appendix A that has signatures of panelists from April 2020. Yet a publication of toxicological studies that you cite in Part 1.3 is from 2021 (i.e., Yigit et al., 2021). Please clarify what your panel considered on this topic and whether that was publicly available.

    i. In Part 6.2.1, you refer to a publication that describes digestibility results described in a publication (i.e., Yigit et al., 2021.). As this publication is post convening of the panel on your behalf, please clarify what your panel considered on this topic at the time point of their signatures and whether that was publicly available.

    j. In Part 6.4 (Assessment of Potential Allergenicity), you refer to a summary of bioinformatics in Appendix E (i.e., Dr. Goodman's unpublished report, completed September 27, 2020) and in Yigit et al., 2021. As this report and publication are post convening of the panel on your behalf, please clarify what your panel considered on this topic at the time point of their signatures and whether that was publicly available.

Please do not send a revised/completely rewritten notice (or any part of the notice, including any Appendix of the notice). As a reminder, confidential data and information cannot be determinant of safety. If you have any questions about the items that require clarification, please let me know. FDA respectfully requests a complete response within 10 business days. If unable to complete the response within that timeframe, please contact me. Thank you.

Sincerely,
Paulette Gaynor

Paulette M. Gaynor, Ph.D.
Senior Policy Advisor

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety, Division of Food Ingredients
U.S. Food and Drug Administration
Tel: 240-402-1192
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formerly Cambridge Crops

March 30, 2022

Dear Dr. Gaynor,

In response to your email sent on Wednesday, March 16, 2022, please find the below answers. FDA's questions are copied below as well in blue text, with Cambridge Crops' answers below FDA's questions in black text.

As always, please feel free to reach out for any further clarifications.

Thank you very much.

Best regards,
Laith

1. On p. 10 (Table 2), you compare the molecular weights of silk fibroin reported in the literature to the molecular weight of silk fibroin that is the subject of GRN 1026 (this notice) and state that based on the SDS-PAGE results, the peak molecular weight (MW) for this silk fibroin is approximately 460 kDa. We note that the MW of this silk fibroin is higher than the MW of the native silk fibroin (heavy chain of ~390 kD and light chain of ~25-26 kD) and the literature MWs listed in Table 2 (390 kDa and 375-446 kDa). We also note that the degumming and dissolution processes lead to different degrees of degradation of native silk fibroin, resulting in lower MWs and different MW distribution of the regenerated silk fibroin, depending on the conditions used for these processes.

    a. Please explain the presence of fragments with MWs greater than that present for the native protein and the literature MWs.

Cambridge Crops' Mori Silk does not contain molecular weight (MW) fragments larger than that present for the native protein and the literature MWs. See our further explanation below in response to Question 1b.

    b. Please clarify if MWs provided in the first paragraph in Part 2.1 on p. 6 are representative of silk fibroin that is the subject of this notice or native silk fibroin and state an average MW and MW range for silk fibroin that is the subject of this notice.

The MWs provided in the first paragraph in Part 2.1 on p. 6 of GRN 1026 represent ranges of MW values estimated by various methods and reported in the published scientific literature for each of the three subunits of native silk fibroin before degumming and solubilization. GRN 1026 cites several review articles reporting these data, including Kaplan and McGrath (2012),[1] Vepari and Kaplan (2007)[2] and Mondal et al. (2007).[3] The highest estimates were calculated from the amino acid sequences of the fibroin

[1] Kaplan D, McGrath K (2012). Protein-Based Materials. Springer Science & Business Media was cited in GRASN 2016; The latest edition of this work was published in 2018, in which the MW data appears on page 177 in Chap 4: Costa F, Silva R, Boccaccini AR (2018). Fibrous protein-based biomaterials (silk, keratin, elastin, and resilin proteins) for tissue regeneration and repair.

[2] Vepari C, Kaplan DL (2007). Silk as a Biomaterial. Prog. Polym. Sci. 32(8-9): 991-1007.

[3] Mondal M, Trivedy K, Kumar SN (2007). The silk proteins, sericin and fibroin in silkworm, *Bombyx mori* Linn., - a Review. Caspian J. Env. Sci. 5(2): 63-76.

polypeptides, which likely reflect most closely the MWs of intact, non-degraded subunits. These values include:

- H-chain: 391 kDa.[4]
- L-chain (including the N-acetyl group): 25.8 kDa.[5]
- P25 protein moiety: 25.179 kDa.[6]

Fully intact native fibroin consists of the H-chain, L-chain (linked together by a single disulfide bond) and the P25 glycoprotein (non-covalently bound to the other proteins) in a molar ratio averaging 6:6:1.[7] Thus, the predicted MW of fibroin is ~445 kDa if one molecule of P25 glycoprotein attached to each fibroin molecule, and ~417 kDa without the P25 glycoprotein attached.[8] The sum of the highest MW estimates of the 3 fibroin subunits reported in the review articles cited on page 6 of GRN 1026 (Bates page 15) is 446 kDa, which is consistent with the predicted maximum MW of the intact fibroin.[9] Further, the accuracy of MW estimation by SDS-PAGE is in the range of 5-10%.[10] As such, the predicted maximum MW of intact fibroin is more accurately represented by 446kDa +/- 10%, which would be ~401-490kDa. This mirrors the Mori Silk figure of 460kDa (which may also be more accurately represented with standard 10% error as 414-506kDa).

It is important to note that there cannot be fragments larger than 391kDa within Mori Silk, as that is the predictive highest MW for the H-chain within fibroin. Smears on SDS-PAGE gels may exist above this value, though, because of the presence of the L-chain (25.8kDa) and P25 protein (25.179kDa). This is not unlike literature values. For example, Pritchard et al. (2013) degummed silkworm cocoons in boiling 0.02 M aqueous Na_2CO_3 for 10, 30, 60, or 90 minutes, air drying overnight and solubilizing each product in 9.3 M aqueous LiBr.[11] SDS-PAGE analysis of the products demonstrated the expected decrease in average MW with increasing degumming time, as evidenced by the clear migration of the smear further down the gel with increasing degumming duration. Silk degummed for 10, 30, 60, and 90 minutes produced a smear in the apparent MW range of 171 to 460 kDa, 31 to 268 kDa, < 171 kDa, and predominantly ≤ 71 kDa, respectively (see Pritchard et al. 2013, page 314, including Figure 1).

Accordingly, GRN 1026 notes that SDS-PAGE analyses of the Mori Silk will yield smears that do not exceed the upper bound of apparent MW on SDS-PAGE gels exceeding that reported in the published scientific literature (i.e., 460 kDa reported by Pritchard et al. 2013).

Finally, the range of average MW values of Mori Silk is be between ~50kDa to ~350kDa.

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[4] Zhou C-Z, Confalonieri F, Jacquet M, Perasso R, Li Z-G, Janin J (2001). Silk Fibroin: Structural Implications of a Remarkable Amino Acid Sequence. PROTEINS: Structure, Function, and Genetics 44: 119-122.

[5] Yamaguchi K, Kikuchi Y, Takagi T, Kikuchi A, Oyama F, Shimura K, Mizuno S (1989). Primary structure of the silk fibroin light chain determined by cDNA sequencing and peptide analysis. J. Molec. Biol. 210(1): 127-139.

[6] Chevillard M, Deleage G, Couble P. (1986). Aminoacid Sequence and Putative Conformational Characteristics of the 25KD Silk Protein of *Bombyx mori*. Sericologia 26(4): 435-449.

[7] Mondal M, Trivedy K, Kumar SN (2007). The silk proteins, sericin and fibroin in silkworm, *Bombyx mori* Linn., - a Review. Caspian J. Env. Sci. 5(2): 63-76.

[8] 391 kDa + 25.8 kDa + 25.179 kDa = 441.978 kDa; 391 kDa + 25.8 kDa= 416.8 kDa.

[9] 390 kDa + 26 kDa + 30 kDa = 446.

[10] https://www.bio-rad.com/en-us/applications-technologies/sds-page-analysis?ID=LW7FGX4VY

[11] Pritchard EM, Hu X, Finley V, Kuo CK, Kaplan DL (2013. Effect of silk protein processing on drug delivery from silk films. Macromol. Biiosci. 13: 311-320.

2. Please confirm that the manufacturing process do not result in formation of silk fibroin nanoparticles. In addition, please provide data demonstrating the particle-size distribution for silk fibroin that is the subject of this notice.

The size distribution of Mori Silk particles does not include detectable levels of particles within the nanoscale range, which we understand from relevant FDA Guidance to be approximately 1 nm to 100 nm in at least one external dimension. [12] We understand that, based on FDA's current Guidance, that developers should consider two (2) key points when determining whether an FDA-regulated product involves nanotechnology:

1. Whether a material or end product is engineered to have at least one external dimension, or an internal or surface structure, in the nanoscale range (approximately 1 nm to 100 nm) and,
2. Whether a material or end product is engineered to exhibit properties or phenomena, including physical or chemical properties or biological effects, that are attributable to its dimension(s), even if these dimensions fall outside the nanoscale range, up to one micrometer (1,000 nm).

As described more fully below, the notified substance has *not* been engineered to have any dimension in the nanoscale range (1nm to 100nm).

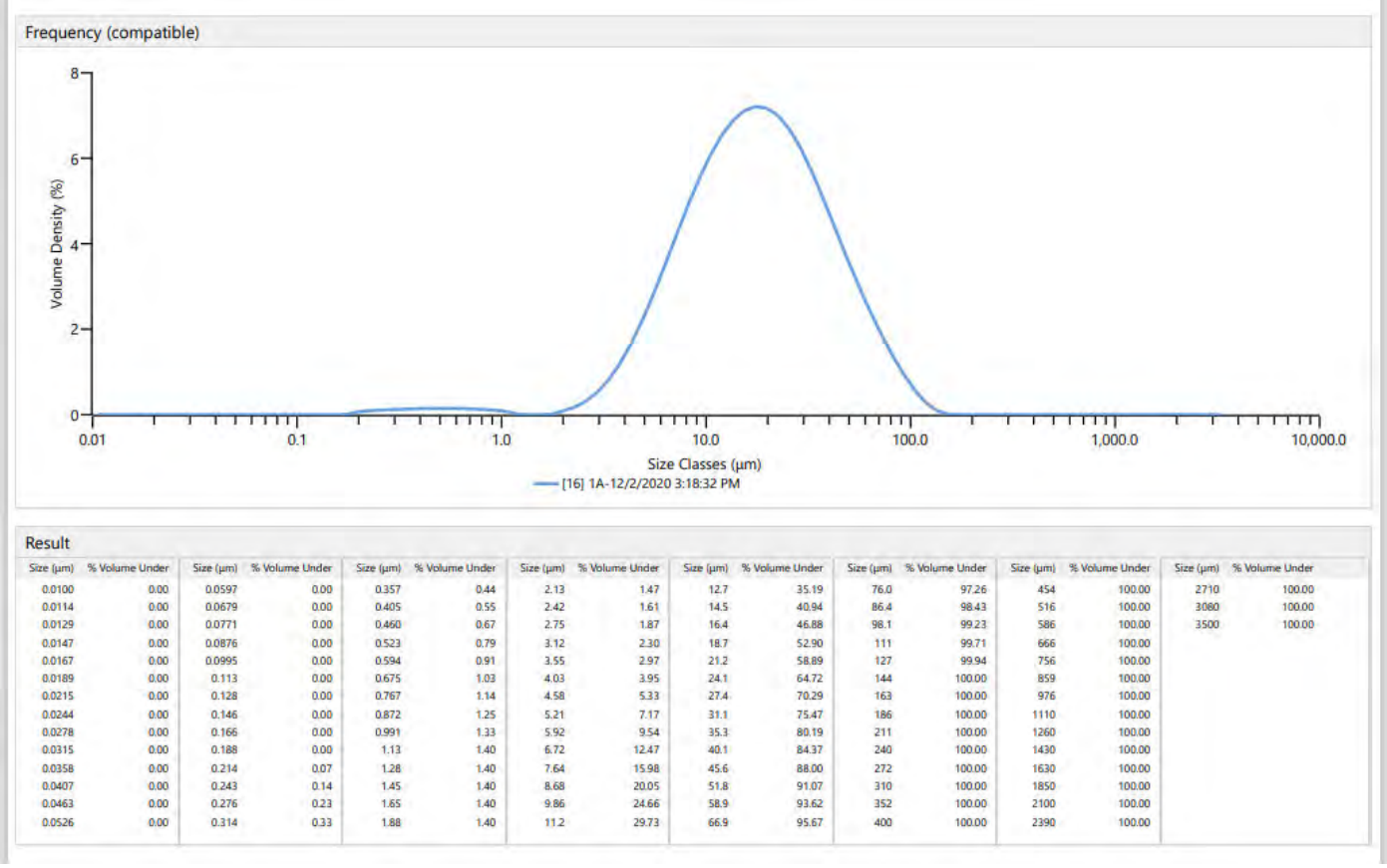
Further, the notified substance has *not* been engineered to exhibit properties or phenomena, including physical or chemical properties or biological effects, that are attributable to its dimensions (which, again, are not nanoscale to the best of our knowledge[13]).

Figure 1, below, presents the typical particle size distribution of Mori Silk, as determined by laser diffraction analysis.[14] Cambridge Crops performed this analysis using a Mastersizer 3000 laser diffraction particle size analyzer. This method is known to provide reliable accuracy and precision in the range of 10nm to 3,500µm. No particles ≤ 168 nm were detected, and <0.07% of the distribution contained incidental particles ~214 nm in size. No particles were detected between ~1130 nm and ~1880 nm. The distribution peaks at around 18,700 nm, and the largest particles are ~144,000 nm in size.

[12] *See e.g.,* FDA's 2014 Guidance: Considering Whether an FDA-Regulated Product Involves the Application of Nanotechnology, available at https://www.fda.gov/media/88423/download

[13] We understand that FDA's Guidance states that "FDA's interest in materials or products 'engineered' to have nanoscale dimensions or related dimension-dependent properties or phenomena is distinct from the more familiar use of biological or chemical substances that may naturally exist at small scales, including at the nanoscale, such as microorganisms or proteins."

[14] Measured using Mastersizer 3000 laser diffraction particle size analyzer; reliable accuracy and precision in the range of 10 nm to 3500 µm.



Result

Size (µm)	% Volume Under	Size (µm)	% Volume Under	Size (µm)	% Volume Under	Size (µm)	% Volume Under	Size (µm)	% Volume Under	Size (µm)	% Volume Under	Size (µm)	% Volume Under	Size (µm)	% Volume Under
0.0100	0.00	0.0597	0.00	0.357	0.44	2.13	1.47	12.7	35.19	76.0	97.26	454	100.00	2710	100.00
0.0114	0.00	0.0679	0.00	0.405	0.55	2.42	1.61	14.5	40.94	86.4	98.43	516	100.00	3080	100.00
0.0129	0.00	0.0771	0.00	0.460	0.67	2.75	1.87	16.4	46.88	98.1	99.23	586	100.00	3500	100.00
0.0147	0.00	0.0876	0.00	0.523	0.79	3.12	2.30	18.7	52.90	111	99.71	666	100.00		
0.0167	0.00	0.0995	0.00	0.594	0.91	3.55	2.97	21.2	58.89	127	99.94	756	100.00		
0.0189	0.00	0.113	0.00	0.675	1.03	4.03	3.95	24.1	64.72	144	100.00	859	100.00		
0.0215	0.00	0.128	0.00	0.767	1.14	4.58	5.33	27.4	70.29	163	100.00	976	100.00		
0.0244	0.00	0.146	0.00	0.872	1.25	5.21	7.17	31.1	75.47	186	100.00	1110	100.00		
0.0278	0.00	0.166	0.00	0.991	1.33	5.92	9.54	35.3	80.19	211	100.00	1260	100.00		
0.0315	0.00	0.188	0.00	1.13	1.40	6.72	12.47	40.1	84.37	240	100.00	1430	100.00		
0.0358	0.00	0.214	0.07	1.28	1.40	7.64	15.98	45.6	88.00	272	100.00	1630	100.00		
0.0407	0.00	0.243	0.14	1.45	1.40	8.68	20.05	51.8	91.07	310	100.00	1850	100.00		
0.0463	0.00	0.276	0.23	1.65	1.40	9.86	24.66	58.9	93.62	352	100.00	2100	100.00		
0.0526	0.00	0.314	0.33	1.88	1.40	11.2	29.73	66.9	95.67	400	100.00	2390	100.00		

Figure 1. Particle Size Distribution of Mori Silk

Based on the analysis conducted by Cambridge Crops, Mori Silk does not contain any nanoscale particles. By way of comparison, we also note that the manufacturing of silk fibroin products containing significant nanoscale particle-size distributions requires substantially more rigorous procedures than the methods employed to manufacture Mori Silk. Zhao and Xie (2015) reviewed the methods promising to produce fibroin nanoparticles for use, for example, in drug-delivery systems.[15] These methods include desolvation (layer-by-layer technique), supercritical fluid technologies, electrospraying, mechanical comminution, and capillary-microdot technique, among others. Gough (2021) noted that electrospinning and related techniques are the most common nanofiber fabrication methods, and that air-spraying and solution spraying are alternative methods that use compressed air as the main driving force, rather than a high-voltage power source.[16] None of these methods are applied in the manufacturing of Mori Silk.

Further, the technical performance of the Cambridge Crops' Mori Silk is enhanced to the extent that the particle size distribution is shifted towards larger particle sizes. Thus, the potential for unintentionally producing nanoscale particles of Mori Silk is minimized through the manufacturing methods for functional and technical reasons.

[15] Zhao Z, Xie M-B (2015). Silk fibroin-based nanoparticles for drug delivery. Int. J. Mol. 16: 4880-4903.
[16] Gough CR (2021). Protein-based nanofibers and thin films for drug delivery applications. Thesis and Dissertations 2935, Rowan University (https://rdw.rowan.edu/etd/2935).

3. On p. 8 (Part 2.4), you state that only food-grade substances and processing aids are used in the manufacture of silk fibroin. Please confirm that alkaline substances used in the degumming process are authorized by a regulation or concluded to be GRAS for such use in the U.S. and that water is the only solvent used in the manufacturing process.

We confirm that the alkaline substances used in the degumming processes are authorized by a regulation or concluded to be GRAS for such use in the US. Cambridge Crops further confirms that water is the only solvent used in its manufacturing process.

4. On p. 8 (Part 2.4), you state that calcium chloride or "other similar GRAS salts" are used to dissolve the degummed silk fibers. Please clarify what other salts besides calcium chloride may be used in the dissolution step.

Salts other than calcium chloride used may include NaCl, $MgCl_2$ or similarly chaotropic salts. Only salts that are affirmed as GRAS as a direct food additive with no limitations other than cGMP for use as a processing aid and pH control agent are used or will be used in the manufacturing of the notified substance.

We also emphasize that the salts are merely a processing aid. Cambridge Crops solubilizes degummed fibroin in an aqueous calcium chloride ($CaCl_2$) solution. Calcium chloride is affirmed as GRAS as a direct food additive with no limitations, other than cGMP, for use as a processing aid and pH control agent, as well as for many other functions in food (21 CFR 184.1193).[17]

Generally, degummed fibroin, which is insoluble in water, can be dissolved in high-ionic strength aqueous or organic salt solutions that disrupt the strong hydrogen bonds that maintain the hydrophobic tertiary structures (i.e., the β-sheet crystallites) of the protein.[18] Lithium bromide (LiBr-H2O) and Ajisawa's reagent ($CaCl_2/H_2O/C_2H_5OH$) are the most common solutions used to degum silk fibroin fibers. There is no reasonable expectation that the salt selection results in any significant differences in fibroin protein outside of making insoluble fibroin become soluble. That said, we do confirm that the manufacturing of the notified substance will *only* include salts that are affirmed as GRAS as a direct food additive with no limitations other than cGMP for use as a processing aid and pH control agent.

5. On p. 8 (Part 2.4), you state that silk fibroin is easily separated from sericin by boiling cocoons in an alkaline solution and thoroughly rinsing with water. On p. 6 (Part 2.1), you note that fibroin and sericin can be separated during the degumming process, and the removal of sericin during the degumming process can be verified. Please discuss the potential for residual sericin being present in the final silk protein or describe how you ensure that it is completely removed from the final silk fibroin.

The sericin family of proteins are generally recognized in peer-reviewed literature as readily removable from silkworm cocoon silk by boiling in alkaline solutions primarily because the sericin proteins

[17] Anticaking agent, antimicrobial agent, curing or pickling agent, firming agent, flavor enhancer, humectant, nutrient supplement, stabilizer and thickener.

[18] Wöltje M, Kölbel A, Aibibu D, Cherif C (2021). A Fast and Reliable Process to Fabricate Regenerated Silk Fibroin Solution from Degummed Silk in 4 Hours. Int. J. Molec. Sci. 22: 10565.

are highly hydrophilic, unlike fibroin.[19] The removal of the sericin proteins permits the fibroin to be reeled. Tables B1 and B2 of GRN 1026 show that the amino acid profile of Cambridge Crops' Mori Silk conforms to the amino acid profile of fibroin reported in the literature. It can be seen from the amino acid compositions that the notified substance does not comprise meaningful sericin amounts, as any significant amount would cause a spike in both serine as well as aspartic acid. Further, it is important to note that residual sericin content is unhelpful technologically in the notified substance, as it may clog purification systems with its tackiness (sericin is known in the industry as "gum," hence the word "degum" to remove sericin).

Specifically, the distinct amino acid profile of the silk sericin proteins is characterized by substantially greater content of serine and aspartic acid residues than the profile of silk fibroin, including the levels of these amino acids reported for silk fibroin in the published scientific literature and the levels measured in Cambridge Crop's Mori Silk (e.g., see Table 2 and Table B1 of GRN 1026). Thus, the untoward presence of sericin in Mori Silk will result in readily detectable elevations of the serine and aspartic acid content of the product.

Further, Fourier Transform Infrared Spectroscopy (FTIR) analysis has been shown to differentiate between degummed silk and sericin standards.[20] In Zhang et al., the authors noted that sericin shows a signature peak in transmission at around 1400cm^{-1}. This is similar to what Cambridge Crops observes in its own FTIR analysis, shown below in Figure 2.

[19] Kunz RI, Costa Brancalhão RM, de Fátima Chasko Ribeiro L, Marçal Natali MR (2016). Silkworm Sericin: Properties and Biomedical Applications. Biomed. Res. Int. Article ID: 8175701 (19 pages); http://dx.doi.org/10.1155/2016/8175701; Kaplin DL, Mello CM, Arcidiacono S, Fossey S, Senecal K, Muller W (1997). Silk. Chapter 4 in Protein-Based Materials, McGrath K and Kaplan D, Birkhäuser, Boston.

[20] Zhang, XM, Wyeth, P (2010). Using FTIR spectroscopy to detect sericin on historic silk. Sci. China Chem. 53, 626-631; https://doi.org/10.1007/s11426-010-0050-y.

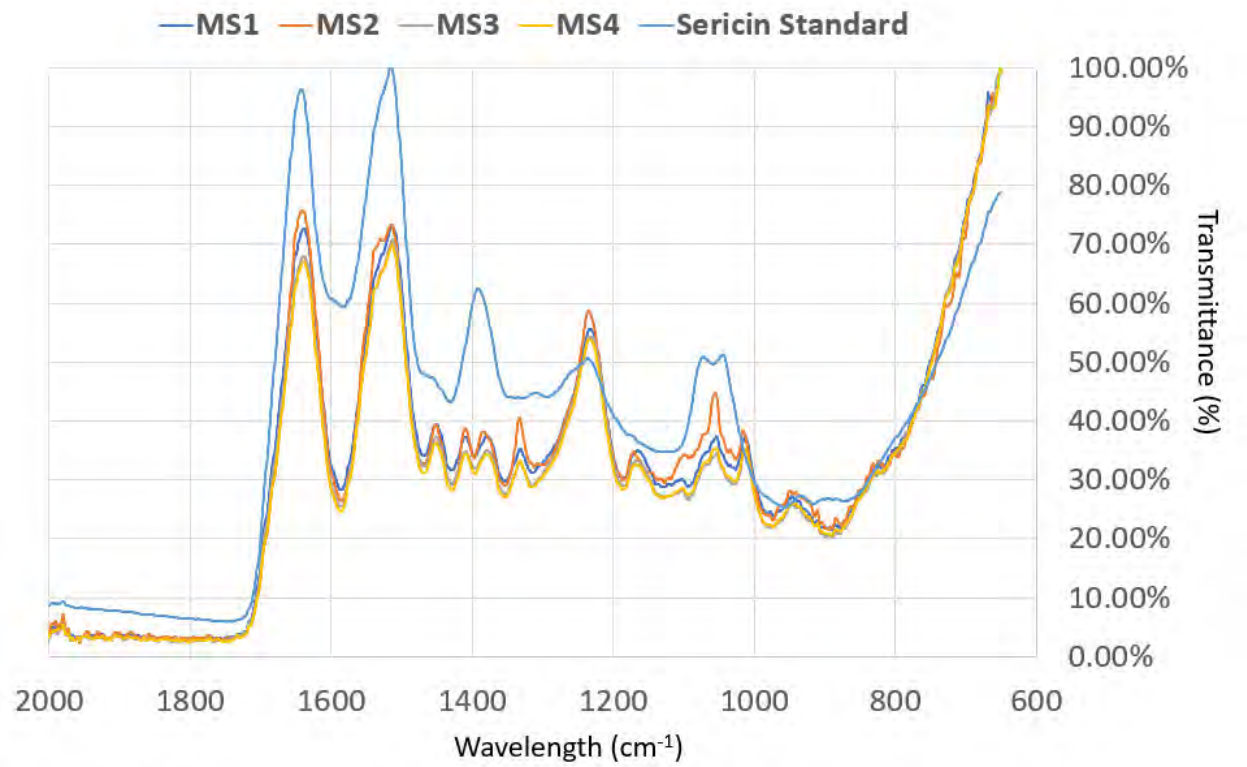


Figure 2. FTIR of four (4) independent Mori Silk manufacturing runs (MS1 through 4) compared to a sericin standard.

As can be seen in Figure 2, above, sericin standards show markedly different results via FTIR. MS1-4 accounts for Mori Silk, with separate independent manufacturing runs (1-4). Cambridge Crops intends on utilizing FTIR at regular intervals, in addition to conducting amino acid analysis at regular intervals, to ensure the removal of sericin.[21]

6. On p. 3 (Table 3a), EPA method 200.8 is identified as the analytical method used to analyze for heavy metals. We note that in the certificates of analysis (Appendix C2), the method was identified as TP-A055. Please clarify this discrepancy.

TP-A055 is the laboratory's technical protocol (TP) number for EPA method 200.5, which is the method that will be used going forward.

[21] As noted in GRN 1026 Appendix A (GRAS Expert Panel Consensus Statement), no instances of allergic episodes from the consumption of silk fibroin were discovered in an extensive literature search. Furthermore, thorough bioinformatics analyses of the full length amino acid sequences of the protein constituents of silk fibers, including sericin and the H-chain, L-chain and P25 glycoprotein of fibroin, confirmed that these constituents do not to pose an allergenicity or allergic cross-reactivity hazard. This is consistent with the absence of any signs of toxicity observed in the repeated oral dose animal tests reported by Yigit et al. (2021) and reviewed in pre-publication reports of these studies by members GRAS Expert Panel for GRN 1026 prior to and during the development and peer review of the manuscript of the Yigit et al. (2021) publication.

7. On p. 3 (Table 3a), you identify USDA/FSIS MLG 8.05 as the method used to test for *Listeria monocytogenes*. We note that this method has been revised and its current version is MLG 8.13 (https://www.fsis.usda.gov/news-events/publications/microbiology-laboratory-guidebook). Please confirm that the correct citation for the method that you use to test for *L. monocytogenes* is MLG 8.13.

Cambridge Crops confirms that the current version of the method, i.e. MLG 8.13, will be used going forward. The batch analysis as shown in GRAS 1026 was conducted with MLG 8.05, which was the applicable method at the time of analysis. However, notifier confirms that it will be using MLG 8.13 going forward.

8. On p. 12, you state silk fibroin consists of 98.6% protein on a dry weight basis. Please describe the composition of the remaining 1.4%.

As indicated on page 12 and in Appendix C3, the nitrogen (N) content analysis of Mori Silk powder produced by spray-drying a 5% solution of the product was 18.44%. The protein content of the product was estimated to be 98.60%, based on a N-to-collagen conversion factor of 5.55 and adjusting for 3.63% moisture content.[22]

As demonstrated in Table 4 of GRN 1026, batch analyses results revealed that the calcium content of 3 nonconsecutive batches of ~5% aqueous Mori Silk ranged from 210 ppm to 216 ppm. On a dry weight basis, the calcium content ranged from 0.397% - 0.572% in these batches.[23] The remaining ~0.908% consists of residual sodium (Na) and chloride (Cl) from the Na_2CO_3 and $CaCl_2$ solutions used to manufacture the product, and potentially other minerals from the use of potable and drinkable Boston city water, such as Mg and Cl.

The only substances used in the manufacturing of Mori Silk are clean *Bombyx mori* cocoons, Boston city water, Na_2CO_3, and $CaCl_2$. Together with the protein content of the product, these substances consistently account for ≥ 99.1%. Importantly, Cambridge Crops utilizes a Hazard Analysis and Risk-Based Preventative Controls plan to ensure manufacturing according to cGMP principles, and there are no other substances expected in the product.

9. On p. 13 (Table 4), the levels of arsenic and lead in each of the three silk fibroin batches are reported to be <0.5 mg/L. We note that the specification limit for each of these heavy metals is <1 mg/L. We request that you reconsider the specification limits for arsenic and lead. The specification limits should reflect the results of batch analyses and be as low as possible.

Cambridge Crops agrees to revise the specification for arsenic and lead from "<1 mg/l" to "<0.5 mg/l."

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[22] The Jones factor, 5.55, is a generally recognized factor for converting measured nitrogen content to total protein content; for example, see Keller S, Liedek A, Shendi D, Bach M, Tovar GEM, Kluger PJ, Southan A (2020). Eclectic characterisation of chemically modified cell-derived matrices obtained by metabolic glycoengineering and re-assessment of commonly used methods. Royal Soc. Chem. 10: 35273.

[23] Batch #342: 210 mg Ca/l ÷ 41,500 mg/l Mori Silk x 100 = 0.506%
Batch #337: 211mg Ca/l ÷ 36,900 mg/l Mori Silk x 100 = 0.572%
Batch #335: 216 mg Ca/l ÷ 54,400 mg/l Mori Silk x 100 = 0.397%
Average: 0.492%

10. On p. 13 (Table 4), the results of batch analyses for *Listeria monocytogenes* and *Salmonella* serovars are reported as "Negative/10g"; however, the specification limits for these microorganisms are stated to be "Negative/25g". Please explain the discrepancy between the sample size identified in the specification limits and in the results of the batch analyses.

Cambridge Crops agrees to revise the specification for *Listeria monocytogenes* and *Salmonella* serovars from "negative/10 g" to "negative/25 g."

11. On p. 13 (Table 4), the value reported for the carbohydrate content in Batch 342 is 0.1%. We believe that the value should be reported as <0.1%. Please confirm that it was an omission and that <0.1% is the correct value for the carbohydrate content in Batch 342.

Cambridge Crops confirms that the missing "<" for Batch 342 is a typographical error and confirms that this was a mistaken omission. The correct value is <0.1% for Carbohydrate content of Batch 342.

In addition, we note that calcium is listed in Table 4 as one of the additional parameters that is measured in silk fibroin. We note that calcium is not listed on p. 12 in Table 3b. Please clarify if calcium is one of the additional parameters that is periodically measured in silk fibroin.

Calcium was inadvertently omitted from Table 3b. Indeed, calcium is one of the additional parameters that is periodically measured in silk fibroin.

12. On p. 2, you state that silk fibroin is used as a coating "to preserve food in accordance with allowed mechanisms described 21 CFR 170.3 (o)" and "intended to extend the shelf life of foods by forming a protective barrier on the outside of the food." On p. 14, you reiterate that silk fibroin is intended to be used as a coating "to preserve food in accordance with allowed mechanisms described in 21 CFR 170.3(o), including as a surface finishing agent." We note that according to 21 CFR 170.3(o), mechanisms to preserve food also include mechanisms that are attributed to antimicrobial agents and antioxidants.

Please clarify if the intended use of silk fibroin includes use as an antimicrobial agent and/or antioxidant. We note that if you intend to use silk fibroin as an antimicrobial agent you would need to provide data demonstrating the antimicrobial effect of silk fibroin in representative foods, including the quantity of silk fibroin required to produce such an effect.

There is no intention to use silk as an antimicrobial agent and/or antioxidant as defined in 21 CFR 170.3(2) and (3), respectively.

13. In Table H1 (Appendix H), you list subcategories of foods in which you intend to use silk fibroin, including fish fillet, shellfish (assumed peeled), and seafood eaten raw. Please describe the specific technical effects of silk fibroin in these food subcategories and state if the intended use of silk fibroin in these foods will result in changes in color, odor, texture and/or flavor that could mislead consumers about the quality (freshness) of these foods or mask deterioration of these foods in a manner that might affect the safe consumption of fish and shellfish.

Mori Silk coating on the surface of foods, including fish fillet, shellfish and seafood eaten raw, is intended to preserve the color, odor, texture, and/or flavor of the finished coated food in a manner that is analogous to physical barriers (e.g., shrink wrap) or food coatings (e.g., protein casings) that provide a

physical barrier to moisture loss from, and gas transmission to, the underlying food. The silk fibroin coating forms an invisible barrier, and does not impart any color or result in changes in any other indicators (e.g., odor, texture, flavor etc.) of the quality (freshness) of fish, shellfish or any other food products so as to mask the deterioration of the food or otherwise to mislead consumers.

In addition, please discuss whether you considered the safety of silk fibroin when it is used on foods that are cooked, fried, or otherwise heated during food preparation.

Cambridge Crops has considered and determined the safety of silk fibroin used on foods that are cooked, fried, or otherwise heated during food preparation. The manufacturing process for Mori Silk involves several steps in which the fibroin is heated to temperatures comparable to those used to heat or cook foods for long durations (i.e., above 60 minutes). As can be seen in Cambridge Crops' safety and digestibility studies, there is no concern with the consumption of silk fibroin at the allotted amounts.

Additionally, the data presented in GRN 1026 demonstrate that the digestibility and amino acid profile of Mori Silk are comparable to those of other commonly consumed dietary proteins, including the proteins of cooked meats and cooked or raw vegetables, for example. Further, there is no expectation that higher temperatures or heat-durations will result in the creation of any unanticipated compounds or proteins.

Thus, the safety of cooked silk fibroin has been considered and was indeed the material within each of Cambridge Crops' safety studies outlined in GRN 1026.

14. On October 6, 2021, you provided an update that the intended use in fish excludes catfish. We note that Table H5 (Appendix H) includes multiple food codes for catfish, indicating that silk fibroin is intended for use as a coating on catfish. We note that all wild-caught and farm-raised *Siluriformes* fish sold for human food are subject to regulation by the U.S. Department of Agriculture (USDA). We request that you address the following:

    a) Please clarify whether silk fibroin is intended for use in catfish. If you intend to use silk fibroin in catfish or in any other foods that are under USDA's jurisdiction, you would need to provide data demonstrating that silk fibroin is suitable for use in such foods.

Cambridge Crops confirms that Mori Silk is not intended for use in catfish and other fish of the *Siluriformes* order. Further, Cambridge Crops confirms that Mori Silk is not intended for use on any USDA-regulated products.

    b) Please clarify whether the food codes corresponding to catfish listed in Table H5 were considered in the dietary exposure assessment. If you do not intend to use silk fibroin in catfish, please provide a revised dietary exposure assessment that excludes uses of silk fibroin in catfish.

As noted above, Cambridge Crops confirms that Mori Silk is not intended for use on any USDA regulated products, including catfish and other fish of the *Siluriformes* order. The inclusion of catfish among the foods considered to contribute to the exposure estimates provided in Table H5 of GRN 1026 results in negligible *overestimates* of dietary exposures to Mori Silk used as intended on foods. There is no safety concern with a higher dietary exposure estimate. In fact, the inclusion of catfish in the dietary exposure assessment means that the assessment included within GRN 1026 is overly conservative as we

anticipated even greater consumption than will actually be the case. Thus, removing the use of Mori Silk on catfish from the exposure estimates would result in slight increases in the margins of exposure, which would not affect the safety conclusion.

Further, we note that a revised calculation will result in considerable cost to the notifier and result only in a negligible change to very slightly *lower* estimates of consumption. However, we emphasize that there Mori Silk is not intended for use in catfish and other fish of the *Siluriformes* order.

15. We note that Table H5 (Appendix H) contains several food codes that represent foods with standards of identity (e.g., chocolate, sweet or dark; cheddar cheese). Please provide a statement that silk fibroin is not intended for use in foods where standards of identity would preclude its use.

Cambridge Crops confirms that silk fibroin is not intended for use in foods where standards of identity preclude its use.

16. On p. 17 (Table 5), you provide the intended use levels of silk fibroin in selected food categories. Please confirm that the use levels are provided based on dehydrated silk fibroin, not the silk fibroin solution.

Cambridge Crops confirms that the use levels are provided based on dehydrated silk fibroin, not silk fibroin solution.

17. On pp. 17-21 (Tables 6-7), you provide the estimates of dietary exposure to silk fibroin. Please confirm that the estimates represent the dietary exposures for the eaters-only population.

Cambridge Crops confirms that the estimates of dietary exposure to silk fibroin (tables 6 through 7 of GRN 1026) represent the dietary exposures for the eaters-only population.

18. We note that the silk fibroin used as a test material in the toxicological studies published by Yigit et al. 2021 was manufactured using lithium bromide in the dissolution step. Please confirm that silk fibroin manufactured using calcium chloride (or other salts that you intend to use) is the same as the test material used in the toxicological studies and that this change in the manufacturing process does not affect your safety conclusion.

As discussed in response to Question 4, there is no reasonable expectation that using $CaCl_2$, NaCl, or $MgCl_2$ in water, rather than some of the solutions of other chaotropic salts commonly used to solubilize fibroin, will result in any significant differences in the product. There is no reason to believe that using $CaCl_2$ rather than LiBr will result in any significant differences in the product. As such, the safety conclusion of Cambridge Crops and the expert panel is unchanged, as the expert panel considered the difference in chaotropic salts as well.

As stated in the response to Question 4, Cambridge Crops will only use salts that are affirmed as GRAS as a direct food additive with no limitations other than cGMP for use as a processing aid and pH control agent are used or will be used in the manufacturing of the notified substance.

19. We note that the GRAS Expert Panel report (Appendix A) includes Tables 1 and 2 containing outdated specifications and dietary exposure estimates. Please provide a statement addressing this discrepancy with the current notice (i.e., GRN 1026) and confirm that it did not affect your safety conclusion.

Cambridge Crops confirms that the revisions to the specifications and estimated daily intakes (EDI) reviewed by the GRAS Expert Panel do not affect the conclusion of safety. The Expert Panel reviewed the information that was provided in GRN 930 and was not reconvened to review GRN 1026. Importantly, the revisions to GRN 930, as reflected in GRN 1026's cover letter, result in lower dietary exposure and narrower specifications than GRN 930 for the very same notified substance. In other words, the expert panel considered a more expansive use of the notified substance. GRN 1026 covers a more limited use, and our reference to the GRAS expert panel report for the broader use scenarios contemplated in GRN 930 is overly conservative. Any discrepancy noted between specifications and dietary exposure estimates referenced in Appendix A (in relation to GRN 930) has no impact on the safety conclusion in GRN 1026, and in fact serves to support the safety of the even lower dietary exposure and narrower specifications noted in GRN 1026.

20. In Part 6.4, you describe proteins of allergenic interest (tropomyosin, arginine kinase, chitinase, paramyosin) identified in the silkworm pupae, cocoons, degummed fibroin, or Mori silk. Further, you include, in Appendix F, a proteomics analysis report (unpublished from Dr. Philp Johnson, including his "expert opinion that the fibroin powder product does not contain Proteins of Allergenic Interest that are detectable using the method employed." Please provide a statement for the record that silk fibroin does not contain any of the proteins of allergenic interest and briefly discuss steps in the method of manufacture that ensure absence of allergenic protein in the silk fibroin ingredient.

Silk fibroin does not contain any of the proteins of allergenic interest.

As noted by the GRAS Expert Panel and published in Yigit et al. (2021), four potentially allergenic silkworm proteins of interest were identified based on the homology of sequences with those of the H-chain, L-chain, P25 glycoprotein and sericin of *Bombyx mori* cocoons, including tropomyosin, arginine kinase, chitinase and paramyosin. As noted in Part 6.4 of GRN 1026, thioredoxin from other insects, but not from *Bombyx mori*, can bind to IgE and was, thus, used as a positive control in the assessment of the potential allergenicity of Mori Silk.

As shown in Part 6.4 and in Appendix F, all of the proteins of allergenic interest and thioredoxin were found at measurable levels in *pupae*—which are not used in the manufacturing process of Mori Silk—but none of these five proteins were present in degummed fibroin or in solubilized Mori Silk powder at or above the limits of detection in the MS method employed. Those methods are discussed further in Appendices E and F (Dr. Goodman and Dr. Johnson reports, respectively). Importantly, we note that Dr. Johnson's conclusions are indeed published in Yigit et al., where Dr. Johnson is a co-author and where the information present within Appendix F was restated and peer-reviewed. Those same opinions and conclusions are also present in the peer-reviewed published abstract and poster for the Food Anaphylaxis Meeting in 2020, where both he and Dr. Goodman are co-authors[24].

[24] Goodman RE, Johnson PE, Yigit S, Sugarman JL, Abu-Taleb LM, Behrens AM (2020). Food allergy safety assessment of extracted silk proteins used in food protection: bioinformatics, mass spectrometry and digestion in

These published analytical results are consistent with the manufacturing process, which uses only the cocoon—and not the eggs or bodies of the larvae, pupae, or adults (i.e. moths) as the source material for manufacturing Mori Silk. The manufacturing process ensures that the insect bodies, whole and parts, are excluded from the process. Silk cocoons that are cleared of the pupae have not been identified as sources of any allergic reactions.[25]

Sericin and other water-soluble denatured proteins are then effectively separated from the insoluble fibroin, which is filtered out of the voluminous degumming solution and rinsed thoroughly with water. The precipitate is then solubilized in heated water with high concentrations of calcium chloride. Following this step, the solution is then purified via filtration membranes to remove the salt and any unwanted insoluble particulates. Following the purification step, the resultant pure fibroin solution is dried at high temperatures to produce Mori Silk powder. These manufacturing steps are listed in Figure 2 of GRN 1026 (Bates page 18).

Through extensive testing at the Harvard Center for Mass Spectrometry and the University of Nebraska-Lincoln (both at the Food Allergy Research and Resource Program (FARRP) as well as the Department of Food Science and Technology), it can be seen that there are no proteins of allergenic interest in silk fibroin. Cambridge Crops will be sending representative batches of Mori Silk to FARRP for periodic liquid chromatography-mass spectroscopy (LC-MS) testing to ensure the absence of proteins of allergenic interest.

Further, there are no major food allergens handled at the manufacturing facility, and there is no risk of allergen cross-contamination based on Cambridge Crop's HARPC analysis and food safety plans.

21. Please address whether silk fibroin, the subject of this GRAS notice, is produced in adult insects; and, please cite the relevant references. You may address the question by addressing the following:

    a) Whether the silk glands producing silk fibroin are only active in larvae or are also active in adult insects.
    b) Whether the silk fibroin-encoding gene/genes are active only in larvae or are also active in adult insects.
    c) **If silk is produced in adult insects**, please address the following:
    d) Are they produced from the same silk gland that produces this protein in larvae?
    e) Distribution of silk fibroin in adult insects, that is, whether it is found in the exoskeleton of the adult insect, or also in non-exoskeletal parts of the insect (including internally).
    f) Identity of silk fibroin in adult insects, that is, whether the adult silk fibroin identical in sequence to the one produced in larvae, and
    g) Relative amounts of silk fibroin (w/w of tissue) in adults compared to that in larvae.

No insects are used—adults or larvae—in Cambridge Crops' manufacturing process. Only cut-and-cleaned cocoons are used, which are devoid from any insects.

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pepsin. Poster presented at: Annual Food Anaphylaxis Meeting as sponsored by the European Academy of Allergy & Clinical Immunology (EEACI), October 16, 2020.

[25] *See* GRAS 1026, Bates page 188, 268.

This is further noted in response to Question 20.

To specifically answer as best as we can: Cui et al. (2018) summarized the biosynthesis silk and fabrication of the silkworm cocoon as follows.[26] Silk fibroin is synthesized in an internal organ called the posterior silk gland (PSG) of silkworms, where it accumulates and is coated with sericin as it passes through the middle silk gland (MSG) and is then secreted via the anterior silk gland (ASG) as the larva spins the silk fibers to fabricate the cocoon.[27] The cocoon is a typical complex trait and the expression of silk protein genes are strictly regulated, subject to time and space constraints.

An extensive search of the literature did not confirm that adult silkworm moths synthesize fibroin.[28,29,30,31]

Further, it is important to note that as stated in Part 5 of GRN 1026, the consumption of *Bombyx mori* insects have only been "presented for background information purposes only," and that the statutory basis from Cambridge Crops' GRAS conclusion is based on scientific procedures to establish the safety of the ingredient based on generally accepted data under the conditions of its intended use in food.

22. GRN 1026 includes a 'GRAS Expert Panel report' (Appendix A). As discussed in the GRAS final rule (81 FR 54960, August 17, 2016), convening such a panel is not required. Nevertheless, as the panel was convened on your behalf, we are seeking clarification about materials that the panel considered given the signatures on the panelists report are from April 2020.

As discussed in response to Question 19, the GRAS Expert Panel reviewed GRN 930 and the safety studies within it. As specified on page 1 of the cover letter from Cambridge Crops to the FDA GRAS Notification Program (7 July 2021), substantive revisions to GRN 930 to produce GRN 1026 include only the following:

- Refinement of exposure estimates based on specific addition levels of the GRAS substance per category of food and the removal of USDA-regulated foods (Part 3.3).
- Reference to published, peer-reviewed report of toxicology and allergenicity studies (Part 6.3, Yigit et al. 2021).
- "Non-Novelty Determinations Regarding Mori Silk™" by Food Directorate, Health Products and Food Branch, Health Canada (June 21, 2021) (Appendix K).

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[26] Cui Y, Zhu Y, Lin Y, Chen L, Feng Q, Wang W, Xiang H (2018). New insight into the mechanism underlying the silk gland biological process by knocking out fibroin heavy chain in the silkworm. BMC Genomics 19: 215.
[27] Long DP, Lu WJ, Zhang Y, Guo Q, Xiang ZH, Zhao AC (2015). New insight into the mechanism underlying fibroin secretion in silkworm. Febs J. 282(1): 89–101.
[28] Xia Q, Li S, Feng Q (2014). Advances in silkworm studies accelerated by the genome sequencing of *Bombyx mori*. Annu. Rev. Entomol. 59: 513–36.
[29] Ma L, Xu HF, Zhu JQ, Ma SY, Liu Y, Jiang RJ, Xia QY, Li S. Ras1(CA) overexpression in the posterior silk gland improves silk yield. Cell Res. 21(6): 934–43.
[30] Sehnal F, Akai H (1990). Insect silk glands: their types, development and function, and effects of environmental factors and morphogenetic hormones on them. Int. J. Insect Morphol. Embryol. 19(2): 79–132.
[31] Jia SH, Li MW, Zhou B, Liu WB, Zhang Y, Miao XX, Zeng R, Huang YP (2007). Proteomic analysis of silk gland programmed cell death during metamorphosis of the silkworm *Bombyx mori*. J. Proteome Res. 6(8): 3003–3010; Montali A, Romanelli D, Cappellozza S, Grimaldi A, de Eguileor M, Tettamanti G (2017). Timing of autophagy and apoptosis during posterior silk gland degeneration in *Bombyx mori*. Arthropod Structure & Develop. 46(4): 518–28.

- Clarification of relevance of cited toxicology studies to Cambridge Crops' exact notified substance (Part 6.3).
- Addition of support vector machine (SVM) bioinformatics searches to investigation of allergenic potential (Part 6.4).
- Refinement of *in vitro* pepsin digestion studies (Part 6.2.1).
- Clarification of manufacturing process including GRAS salts as process aids (Part 2.4).
- Refinement of heavy metals specifications (Part 2.5).
- Citation of standard analytical methods for specification parameters and batch analysis results (Part 2.5).

Furthermore, Dr. Richard Goodman, who served as a GRAS Expert Panelist also co-authored Yigit et al. (2021). None of the differences between GRN 930 and GRN 1026 warrant revising the panel's analysis or conclusions.

a) In Part 1.5, you refer to Appendix A that has signatures of panelists from April 2020. Yet a publication of toxicological studies that you cite in Part 1.3 is from 2021 (i.e., Yigit et al., 2021). Please clarify what your panel considered on this topic and whether that was publicly available.

The GRAS Expert Panel considered materials that were presented in GRN 930 and the associated safety studies. The FDA Review Team for GRN 930 recommended that Cambridge Crops narrow some of the specifications and lower dietary exposure estimates and publish the safety studies in a peer-reviewed scientific journal.

All of FDA's recommendations were achieved, including narrowing specifications, refining (lowering) exposure estimates, conducting SVM analyses, and publication of the safety studies (Yigit et al. 2021). Cambridge Crops did not reconvene the Expert Panel because all of the seminal data presented to the Expert Panel in the GRN 930 dataset, which served as the basis for the Panel's conclusions, were unchanged in GRN 1026. Other revisions incorporated into GRN 1026 only bolstered the Panel's conclusions.

Please note, as well, that a member of the GRAS Expert Panel, Dr. Richard Goodman, co-authored Yigit et al. (2021). Another co-author was Dr. Philip Johnson, who contributed his analysis presented in Appendix F.

b) In Part 6.2.1, you refer to a publication that describes digestibility results described in a publication (i.e., Yigit et al., 2021). As this publication is post convening of the panel on your behalf, please clarify what your panel considered on this topic at the time point of their signatures and whether that was publicly available.

Dr. Richard Goodman led and evaluated the digestibility studies presented in GRN 930. The FDA Review Team for GRN 930 recommended the inclusion of a digestibility test using an additional pepsin:protein ratio. This additional test was completed after convening of the Expert Panel and the signing of their evaluation of the GRN 930 dataset, and was subsequently incorporated into the Yigit et al. (2021) publication. Cambridge Crops did not reconvene the Expert Panel after the completion of this additional test because the results supported and bolstered the Expert Panel's evaluation and conclusions based on the GRN 930 dataset.

c) In Part 6.4 (Assessment of Potential Allergenicity), you refer to a summary of bioinformatics in Appendix E (i.e., Dr. Goodman's unpublished report, completed September 27, 2020) and in Yigit et al., 2021. As this report and publication are post convening of the panel on your behalf, please clarify what your panel considered on this topic at the time point of their signatures and whether that was publicly available.

The GRAS Expert Panel's evaluation considered all of the data presented in GRN 930, which did not include the new test using and additional pepsin:protein ratio or the SVM analyses recommended by FDA's GRN 930 Review Team. However, the additional test and analyses were incorporated into the Yigit et al. (2021) publication, which was co-authored by Dr. Goodman, and the results bolstered the Panel's conclusions. Thus, Cambridge Crops did not reconvene the Panel.

As always, please feel free to reach out if there are any questions.

Laith Abu-Taleb
laith@mori.com

From: Laith Abu-Taleb
To: Gaynor, Paulette M
Subject: [EXTERNAL] Re: GRN 1026 amendment – items for clarification
Date: Thursday, May 5, 2022 4:51:44 PM
Attachments: cid%3Aimage001.png%4001D1C57E.DFA022A0
cid%3Aimage002.jpg%4001D1C57E.DFA022A0
cid%3Aimage003.jpg%4001D1C57E.DFA022A0
cid%3Aimage004.jpg%4001D1C57E.DFA022A0
cid%3Aimage005.jpg%4001D1C57E.DFA022A0
cid%3Aimage006.jpg%4001D1C57E.DFA022A0
2022.05.05.Re GRN 1026_Mori Silk.pdf
Pritchard 2013.pdf
Analytical Results.pdf

Dear Dr. Gaynor,

We hope you and the team are doing well.

Please find attached our answers to your questions sent to us via email on April 20, 2022. If there are any further questions, please do let me know.

Thank you very much.

Best regards,
Laith

On Wed, Apr 20, 2022 at 2:24 PM Gaynor, Paulette M <Paulette.Gaynor@fda.hhs.gov> wrote:

> Laith M. Abu-Taleb, Esq.
>
> Cambridge Crops, Inc. d/b/a Mori
>
> *By email*: laith@mori.com
>
> Dear Mr. Abu-Taleb,
>
> As we continue with our evaluation of GRN 1026, including the amendment that you sent on March 30, 2022, we have identified items that require clarification. These items follow:
>
> 1. In the response to Question 1b, you referred to the information on page 314 in the

article by Pritchard *et al*. 2013. We note that the article does not have a page 314. Please provide the correct page number.

2. In the response to Question 2, you stated that no particles ≤168 nm were detected; however, based on Figure 1 included in your response, no particles ≤188 were detected. Please clarify this.
3. In the response to Question 10, you stated that you agree to revise the specification limits for *Listeria monocytogenes* and *Salmonella* serovars from "negative/10 g" to "negative/25 g". We note that the specification limits for these microorganisms provided in GRN 001026 are set to "negative/25 g" and as such do not need to be revised. However, the results of batch analyses that you provided are reported for 10 g samples.
    a. Please clarify why the results of batch analyses were reported for 10 g samples if the specification limits for both microorganisms were established based on a 25 g sample.
    b. We request that you provide the results for *Salmonella* serovars in a 25 g sample from analysis of three nonconsecutive batches.
    c. We note that the USDA/FSIS MLG 8.05 (Isolation and Identification of Listeria monocytogenes from Red Meat, Poultry, Ready-To-Eat, Siluriformes (Fish) and Egg Products, and Environmental Samples) is not suitable to test for *L. monocytogenes* in silk fibroin. Considering the above, we request that you provide the analysis results for *L. monocytogenes* from three nonconsecutive batches conducted using an appropriate method and sample size. For example, the BAM Chapter 10 method is the FDA-accepted method applicable to foods in general.
4. In the response to Question 12, you stated that silk fibroin is not intended to be used as an antimicrobial agent and/or antioxidant as defined in 21 CFR 170.3(2) and (3). We note that the definitions of antimicrobial agents and antioxidants are provided in 21 CFR 170.3**(o)**(2) and (3). Please confirm that you were referring to 21 CFR 170.3**(o)**(2) and (3) in your response.

Please do not send a revised/completely rewritten notice (or any part of the notice, including any Appendix of the notice). As a reminder, confidential data and information cannot be determinant of safety. If you have any questions about the items that require clarification, please let me know. FDA respectfully requests a complete response within 10 business days. If unable to complete the response within that timeframe, please contact me. Thank you.

Sincerely,

Paulette Gaynor

Paulette M. Gaynor, Ph.D.
Senior Policy Advisor

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety, Division of Food Ingredients
U.S. Food and Drug Administration
Tel: 240-402-1192
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--

Laith Abu-Taleb, Chief Strategy Officer & General Counsel | www.mori.com | (c) +1.202.834.3174 | laith@mori.com | Bio

formerly Cambridge Crops

May 5, 2022

Dear Dr. Gaynor,

In response to your email sent on Wednesday, April 20, 2022, please find the below answers. FDA's questions are copied below as well in blue text, with Cambridge Crops' answers below FDA's questions in black text.

As always, please feel free to reach out for any further clarifications.

Thank you very much.

Best regards,
Laith

1. In the response to Question 1b, you referred to the information on page 314 in the article by Pritchard *et al*. 2013. We note that the article does not have a page 314. Please provide the correct page number.

Attached is a copy of the article by Pritchard *et al*. with the journal page numbering including page 314 used in the reference cited in Question 1b.

2. In the response to Question 2, you stated that no particles ≤168 nm were detected; however, based on Figure 1 included in your response, no particles ≤188 were detected. Please clarify this.

You are correct. No particles ≤188 were detected, as can be seen from Figure 1 from Cambridge Crops' March 30th letter to FDA. This was a typo in our previous answer, our apologies.

3. In the response to Question 10, you stated that you agree to revise the specification limits for *Listeria monocytogenes* and *Salmonella* serovars from "negative/10 g" to "negative/25 g". We note that the specification limits for these microorganisms provided in GRN 001026 are set to "negative/25 g" and as such do not need to be revised. However, the results of batch analyses that you provided are reported for 10 g samples.
    a. Please clarify why the results of batch analyses were reported for 10 g samples if the specification limits for both microorganisms were established based on a 25 g sample.

The results were reported based on the method that was used for the analysis reported in GRN001026. This has been corrected and the most current analytical results, which are attached, are reported based on a 25 gram sample.

    b. We request that you provide the results for *Salmonella* serovars in a 25 g sample from analysis of three nonconsecutive batches.

Analytical results for *Salmonella* serovars in a 25 g sample, from three nonconsecutive batches (each batch around three months apart), are attached.

    c. We note that the USDA/FSIS MLG 8.05 (Isolation and Identification of Listeria monocytogenes from Red Meat, Poultry, Ready-To-Eat, Siluriformes (Fish) and Egg Products, and Environmental Samples) is not suitable to test for *L. monocytogenes* in silk

fibroin. Considering the above, we request that you provide the analysis results for *L. monocytogenes* from three nonconsecutive batches conducted using an appropriate method and sample size. For example, the BAM Chapter 10 method is the FDA-accepted method applicable to foods in general.

Analytical results for *L. monocytogenes* using BAM Chapter 10 for three nonconsecutive batches (each batch approximately 3 months apart) are attached.

Please note that the analytical lab, Eurofins, conducted both BAM Chapter 10 as well as AOAC-RI 061702 methods on one of the batches (Batch Number 20220413). The AOAC-RI 061702 method results for that batch are reported on Page 5 of the attached Analytical Results PDF, and the BAM Chapter 10 method results for that batch are reported on page 7.

To further clarify and simplify the analytical results within the attachments, we provide the following table alongside the representative page numbers within the attached Analytical Results PDF.

Sample code / Batch Number	Date of Manufacture	Listeria FDA BAM Chapter 10	Salmonella AOAC-RI 121501
20211013	Oct. 13, 2021	Not detected/25 g *(PDF page 1)*	Not detected/25 g *(PDF page 1)*
20220119	Jan. 19, 2022	Not detected/25 g *(PDF page 3)*	Not detected/25 g *(PDF page 3)*
20220413	Apr. 13, 2022	Not detected/25 g *(PDF page 7)*	Not detected/25 g *(PDF page 5)*

4. In the response to Question 12, you stated that silk fibroin is not intended to be used as an antimicrobial agent and/or antioxidant as defined in 21 CFR 170.3(2) and (3). We note that the definitions of antimicrobial agents and antioxidants are provided in 21 CFR 170.3(o)(2) and (3). Please confirm that you were referring to 21 CFR 170.3(o)(2) and (3) in your response.

We confirm that we were referring to 21 CFR 170.3(o)(2) and (3) in our response.

Once again, thank you very much. As always, please feel free to reach out if there are any questions.

Best regards,

Laith Abu-Taleb
laith@mori.com

Ten pages have been removed in accordance with copyright laws. The removed reference citation is:

E. M. Pritchard, X. Hu, V. Finley, C. K. Kuo, and D. L. Kaplan, “Effect of silk protein processing on drug delivery from silk films,” *Macromolecular Bioscience*, vol. 13, no. 3, pp. 311–320, 2013.

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Mori

Catherine Wagner
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Client Code: UC0000312

ANALYTICAL REPORT

AR-22-UC-006139-04
Report Supersedes AR-22-UC-006139-03

Received On: 27Apr2022
Reported On: 05May2022

Eurofins Sample Code:	126-2022-04270129	**Sample Registration Date:**	27Apr2022
Client Sample Code:	20211013	**Condition Upon Receipt:**	acceptable, 19.2°C
Sample Description:	Mori Silk	**Sample Reference:**	

UM2N6 - Listeria spp. - BAM Chapter 10	**Reference**	**Accreditation**	**Completed**
	FDA BAM Chapter 10	ISO/IEC 17025:2017 A2LA 3329.08	02May2022

Parameter	**Result**
Listeria Species	Not Detected per 25 g

UMDTC - Salmonella spp. - AOAC-RI 121501	**Reference**	**Accreditation**	**Completed**
	AOAC-RI 121501	ISO/IEC 17025:2017 A2LA 3329.08	28Apr2022

Parameter	**Result**
Salmonella	Not Detected per 25 g

Comments:
Corrected client sample name and sample description.

Respectfully Submitted,

Jordan Ramsby
Deputy Supervisor and Team Leader

Mori

Catherine Wagner
500 Rutherford Ave
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Client Code: UC0000312

ANALYTICAL REPORT

AR-22-UC-006139-04
Report Supersedes AR-22-UC-006139-03

Received On: 27Apr2022
Reported On: 05May2022

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ANALYTICAL REPORT

AR-22-UC-006140-04
Report Supersedes AR-22-UC-006140-03

Received On: 27Apr2022
Reported On: 05May2022

Eurofins Sample Code:	126-2022-04270130	**Sample Registration Date:**	27Apr2022
Client Sample Code:	20220119	**Condition Upon Receipt:**	acceptable, 19.2°C
Sample Description:	Mori Silk	**Sample Reference:**	

UM2N6 - Listeria spp. - BAM Chapter 10	**Reference**	**Accreditation**	**Completed**
	FDA BAM Chapter 10	ISO/IEC 17025:2017 A2LA 3329.08	02May2022

Parameter	**Result**
Listeria Species	Not Detected per 25 g

UMDTC - Salmonella spp. - AOAC-RI 121501	**Reference**	**Accreditation**	**Completed**
	AOAC-RI 121501	ISO/IEC 17025:2017 A2LA 3329.08	28Apr2022

Parameter	**Result**
Salmonella	Not Detected per 25 g

Comments:
Corrected client sample name and sample description.

Respectfully Submitted,

[redacted]

Jordan Ramsby
Deputy Supervisor and Team Leader

Mori

Catherine Wagner
500 Rutherford Ave
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Client Code: UC0000312

ANALYTICAL REPORT

AR-22-UC-006140-04
Report Supersedes AR-22-UC-006140-03

Received On: 27Apr2022
Reported On: 05May2022

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ANALYTICAL REPORT

AR-22-UC-005769-04
Report Supersedes AR-22-UC-005769-03

Received On: 25Apr2022
Reported On: 05May2022

Eurofins Sample Code:	126-2022-04250016	**Sample Registration Date:**	25Apr2022
Client Sample Code:	20220413	**Condition Upon Receipt:**	acceptable, 19.5°C
Sample Description:	Mori Silk	**Sample Reference:**	

UMDTC - Salmonella spp. - AOAC-RI 121501	**Reference**	**Accreditation**	**Completed**
	AOAC-RI 121501	ISO/IEC 17025:2017 A2LA 3329.08	26Apr2022

Parameter	**Result**
Salmonella	Not Detected per 25 g

UMQDX - Listeria spp. - AOAC-RI 061702	**Reference**	**Accreditation**	**Completed**
	AOAC-RI 061702	ISO/IEC 17025:2017 A2LA 3329.08	26Apr2022

Parameter	**Result**
Listeria Species	Not Detected per 25 g

Comments:
Corrected client sample name and sample description.

Respectfully Submitted,

[redacted]

Jordan Ramsby
Deputy Supervisor and Team Leader

Mori

Catherine Wagner
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ANALYTICAL REPORT

AR-22-UC-005769-04
Report Supersedes AR-22-UC-005769-03

Client Code: UC0000312

Received On: 25Apr2022
Reported On: 05May2022

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Client Code: UC0000312

ANALYTICAL REPORT

AR-22-UC-006141-04
Report Supersedes AR-22-UC-006141-03

Received On: 27Apr2022
Reported On: 05May2022

Eurofins Sample Code:	126-2022-04270236	**Sample Registration Date:**	27Apr2022
Client Sample Code:	20220413	**Condition Upon Receipt:**	acceptable, 20.4°C
Sample Description:	Mori Silk	**Sample Reference:**	MSPR001 DOM: 20220413

UM2N6 - Listeria spp. - BAM Chapter 10	**Reference**	**Accreditation**	**Completed**
	FDA BAM Chapter 10	ISO/IEC 17025:2017 A2LA 3329.08	02May2022

Parameter	**Result**
Listeria Species	Not Detected per 25 g

Comments:
Corrected client sample name and sample description.

Respectfully Submitted,

[redacted]

Jordan Ramsby
Deputy Supervisor and Team Leader