Bayer CropScience

PAT/*pat* PROTEIN

IN VITRO DIGESTIBILITY STUDY IN

HUMAN SIMULATED GASTRIC FLUID

DATA REQUIREMENT
No applicable guidelines

REPORT OF STUDY SA 09048
Sponsor identification number: Lynx-PSI N°TX99L074

AUTHOR / STUDY DIRECTOR: [redacted]

TESTING FACILITY:

Bayer CropScience
355, rue Dostoïevski
BP 153
06903 Sophia Antipolis Cedex
France

SPONSOR:

Bayer AG
Bayer CropScience
Alfred Nobel Str. 50
40789 Monheim
Germany

STUDY COMPLETED ON: JUNE 26, 2009

STATEMENT OF NO DATA CONFIDENTIALITY CLAIM

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA § 10 (d) (1) (A), (B) or (C).

Company Name:

Company Agent:

Title:

Signature:

Date: _____

These data are the property of Bayer CropScience, and as such, are considered to be confidential for all purposes other than compliance with FIFRA § 10. Submission of these data in compliance with FIFRA does not constitute a waiver of any right to confidentiality which may exist under any other statute or in any other country.

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The study here reported was performed in accordance with the principles of Good Laboratory Practice ("Bonnes Pratiques de Laboratoire") described in the following issue, with the exception of the test item solution, which was not analyzed for concentration, homogeneity and stability, as well as the primary antibody directed against the PAT/pat protein which has no batch number and no expiry date.

- Organization for Economic Cooperation and Development (O.E.C.D.) Principles of Good Laboratory Practice, 1997 (January 26, 1998).
- European Directive 2004/10/EC (February 11, 2004).
- French Decree N°2006-1523, regarding Good Laboratory Practice (December 04, 2006).
- U.S. Environmental Protection Agency (E.P.A.)
  40 CFR part 160
  Federal Insecticide, Fungicide and Rodenticide Act (FIFRA);
  Good Laboratory Practice Standards: Final Rule, August 17, 1989.
- Good Laboratory Practice Standards for Toxicology studies on Agricultural Chemicals, Ministry of Agriculture, Forestry and Fisheries (M.A.F.F.) in Japan, notification 11 Nousan N°6283, October 01, 1999, modified by: notification 12 Nousan n°8628, December 06, 2000.

Author / Study Director: [redacted]

Date: June 26, 2009

Sponsor Representative: [redacted]

Date: June 29, 2009

Study Submitter:

Date:

FLAGGING STATEMENTS

This page is reserved for flagging statements as may be required by E.P.A.

QUALITY ASSURANCE STATEMENT

The conduct of the study has been subjected to periodic inspections by the Bayer CropScience Sophia Antipolis Quality Assurance Unit. The types and dates of inspections and dates of reporting to Study Director and management are given below:

Type of Q.A. inspection	Study phases inspected	Date of Q.A. inspection	Date of reporting to Study Director	Date of reporting to Management
Study-based	Study plan	March 16, 2009	March 16, 2009	March 27, 2009
Process-based	Migration Western blot	March 18, 2009 April 02, 2009	March 18, 2009 April 06, 2009	March 27, 2009 April 08, 2009
Study-based	Final report	June 22, 2009	June 22, 2009	June 23, 2009

This report has been audited by Quality Assurance personnel in accordance with the appropriate standardized operating methods. The reported results accurately reflect the original data of the study.

Quality Assurance Group Leader: [redacted]

Date: June 26, 2009

SIGNATURE

I, the undersigned, hereby declare that the work was performed under my supervision according to the procedures described and that this report provides a correct and faithful record of the results obtained.

There were no circumstances which affected the quality and integrity of the data.

Author / Study Director:

[redacted]

Date: June 26, 2009

STUDY PROFESSIONALS

The following professionals were involved in the conduct of this study:

STUDY DIRECTOR	:	[redacted]
REPLACEMENT STUDY DIRECTOR	:	[redacted]
RESPONSIBLE TECHNICIAN	:	[redacted]
REPORT UNIT ASSISTANT	:	[redacted]

TABLE OF CONTENTS

SUMMARY

The PAT/*pat* protein (encoded by the *pat* gene, produced in *E. coli*) was tested for digestive stability in human simulated gastric fluid (SGF) after incubation times of 0.5 to 60 minutes. The protocol was in accordance with Thomas *et al*. (2004) protocol (1).

Test protein or reference protein solutions were incubated with human SGF (a pepsin solution at pH 1.2) at approximately 37°C and aliquots were taken for analysis at time-points of 0, 0.5, 2, 5, 10, 20, 30 and 60 minutes. The samples were analysed for presence of the test protein and potential stable protein fragments by Coomassie blue-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot. The immunodetection was performed using a polyclonal antibody directed against the PAT/*pat* protein. Appropriate controls included the test protein without pepsin at pH 1.2 (0 and 60 minutes time-points), SGF alone (0 and 60 minutes time-points), and a 10% loading test protein control. Reference proteins horseradish peroxidase (HRP) and ovalbumin (OVA), known to be digested rapidly and slowly, respectively, were tested in parallel.

The PAT/*pat* protein was degraded very rapidly in human simulated gastric fluid, within 0.5 minutes of incubation, in presence of pepsin, at pH 1.2.

INTRODUCTION

This *in vitro* human simulated gastric fluid (SGF) digestibility study has been conducted to provide a full GLP study carried out according to a current internationally recognized protocol. This study follows the protocol and the methods used in the International Life Science Institute (ILSI) ring trial (1). The method is based on the United States *Pharmacopeia* (2).

In this study the test material was the PAT/*pat* protein (encoded by the *pat* gene, produced in *E. coli*). Two control proteins, horseradish peroxidase (HRP) and ovalbumin (OVA), were included in this study. They were chosen as they are known to be rapidly and slowly digested, respectively (1).

The study protocol is presented in Attachment 1.

The study schedule was as follows:

Study initiation date*	March 13, 2009
Sponsor representative protocol approval date	March 13, 2009
Experimental starting date	March 18, 2009
Experimental completion date	April 03, 2009

* Date of protocol approval by Study Director

MATERIAL AND METHODS

1 - PROTEINS AND PEPSIN

The certificates of analysis are presented in Attachment 2.

1.1 Test protein

The test item PAT/*pat* protein was supplied by BioAnalytics (Bayer CropScience NV, Swijnaarde, Belgium).

Identification ..... :	PAT/*pat* protein (encoded by the *pat* gene, produced in *E. coli*)
Batch N° ..... :	ADW040708
Description ..... :	Lyophylized powder
Purity ..... :	87.5 %
Storage ..... :	Dry and dark at room temperature
Certified through .... :	July 2009

1.2 Reference proteins

The control proteins were purchased from Sigma, France.

Identification ..... :	Horseradish peroxidase
Reference ..... :	P6782
Batch N° ..... :	125K7415
Enzymatic activity.. :	1080 units/mg Solid (using ABTS);
.....	298 units/mg Solid (using Pyrogallol)
Description ..... :	Red-brown powder
Storage ..... :	Approximately +4°C
Certified through .... :	February 2011

Identification ..... :	Ovalbumin (OVA), albumin from chicken egg white
Reference ..... :	A5503
Batch N° ..... :	126K7009
Description ..... :	White powder
Purity ..... :	>99 %
Storage ..... :	Approximately +4°C
Certified through .... :	March 2012

1.3 Proteolytic enzyme

Identification ..... :	Pepsin (from porcine gastric mucosa)
Source..... :	Sigma, Reference P6887
Batch N° ..... :	056K767622
Description ..... :	White lyophilized powder
Activity..... :	3260 Units/mg protein
Purity ..... :	92 %
Storage ..... :	Approximately -20 °C
Certified through .... :	June 2009

2 - PROTEIN SOLUTIONS

The test protein was solubilized in Tris 20mM pH 7.5, 5 mM EDTA at the final concentration of 2.5 mg PAT/*pat* protein/ml. The two reference proteins were solubilized at 2.5 mg /ml in the same buffer.

3 - TEST SYSTEM

The human simulated gastric fluid (SGF) was prepared as follows:

- Preparation of G-con solution (2 mg/ml NaCl, pH 1.2).
- Addition of the pepsin to the G-con solution. The quantity of pepsin in the SGF solution was calculated so that the final concentration of pepsin in the digestion tubes was of 10 activity units per µg of test protein.

In order to validate the activity of the pepsin solution, the reference proteins HRP and OVA were tested concurrently.

4 - DIGESTION INCUBATION

The protein incubation for the test and reference materials was made in 2 ml microcentrifuge tubes in a waterbath at approximately 37°C. For each test and reference protein solution, 80 µl was added to 1520 µl of SGF and mixed. Samples of 200 µl were taken after 0.5, 2, 5, 10, 20, 30 and 60 minutes incubation. The tubes were agitated before each sampling and at approximately 45 minutes.
A dilution of the test protein solution at 1/10 in Tris 20mM pH 7.5, 5 mM EDTA buffer was prepared for the 10% loading control.
For the control tubes (G-con + protein time 60 min and SGF alone, 60 min), each containing 200 µl of sample, the neutralisation solution (70 µl 200 mM NaHCO3 pH 11.0) was added directly to the incubation tubes.
For the serial digestion samples and the other control tubes, the reaction was terminated by adding the 200 µl of digestion sample to a tube containing 70 µl of 200 mM NaHCO3 (pH 11.0).

Additional control samples were prepared:
- a zero minute incubation of protein (10 µl) with 'SGF without pepsin' (190 µl);
- a zero minute incubation of the 1/10 diluted protein (10 µl) with 'SGF without pepsin' (190 µl) (10% loading control);
- a 60 minutes incubation of protein (10 µl) with 'SGF without any pepsin' (190 µl);
- a 'time zero' sample was produced by adding the protein (10 µl) to SGF (190 µl) after the reaction was terminated as above;
- a sample of SGF alone before incubation and the reaction terminated as above;
- a sample of SGF alone after 60 minutes incubation and the reaction terminated as above.

Aliquots of 20 µl of the samples were used for analysis on SDS-PAGE gels and the remaining was frozen at -20°C.

5 - SDS-PAGE ANALYSIS

Gel electrophoresis was carried out following the method of Laemmli (3) using a Bio-Rad Mini-Protean III cell (Bio-Rad, France). Prior to running SDS-PAGE, 5 µl of 5X Laemmli solution was added (supplemented with few grains of sucrose) to 20 µl of digestion samples and heated for 10 minutes at more than 90°C before loading the gel. Samples of 15 µl were added to wells of an SDS-PAGE gel (15 well, 1 mm 10-20% gradient polyacrylamide Tris/Tricine, run with 1X Tris/Tricine Buffer) (Bio-Rad, France).

A suitable marker solution (2.5-200 kDa) was used to provide reference points of known molecular weights on the gel (Mark 12, Invitrogen, France). In addition, the Kaleidoscope, prestained standard molecular weight marker (Bio-Rad, France) was included on the gel. Both markers were prepared by mixing 5µl ready-made marker with 10 µl of 5X Laemmli solution.

The electrophoresis was carried out with a constant voltage set at 100 volts until the bromophenol line has reached the bottom of the gel.

The gels were stained by the Coomassie blue method (Invitrogen) based on the work of Neuhoff *et al.* (4). After rinsing, the gels were scanned using a GS800 scanner (Bio-Rad). The gels are not stable for more than two weeks, so the raw data are the scanned image.

In addition, a second SDS-PAGE was used for the western blot analysis. This gel was loaded with approximately 200 times less protein in quantity to take into account the better level of detection of the western blot technique compared to one of Coomassie blue staining.

6- WESTERN BLOT ANALYSIS

A polyvinylidene fluoride (PVDF) membrane was placed on the SDS-PAGE gel in a Tris/Glycine buffer and an electrical current applied in order to transfer the protein bands onto the membrane. To detect the PAT/*bar* protein band and/or its potential fragments, the membrane was incubated in the presence of a specific rabbit polyclonal anti-PAT/*bar* protein antibody.

The hybridization of the antibody with the proteins immobilized on the membrane was revealed using a goat anti-rabbit polyclonal antibody coupled with a peroxidase. The hybridization bands were visualized using enhanced chemiluminescence (ECL) detection system (GE Healthcare Life Sciences, France).

The autoradiographs were scanned using a GS800 scanner (Bio-Rad) and resulting image was retained in the raw data and reported in the final report.

7 - DATA STORAGE

All raw data, supporting documents as well as protocol, aliquot of the test substance solution and final report are maintained in the archive room.
All of the above will be archived for at least 10 years in the designated areas at:

Bayer CropScience
355, rue Dostoïevski
BP 153
06903 Sophia Antipolis Cedex
France

RESULTS AND DISCUSSION

1 - PAT/*pat* protein (Fig. 1,4)

The SDS-PAGE analysis of the PAT/*pat* protein solution (Fig. 1) showed one major band located close to the molecular weight marker at 21.5 kDa, in accordance with the molecular weight of the PAT/*pat* protein (5). The 10% PAT/*pat* protein control was also visible at approximately 21.5 kDa with lower intensity compared to the undiluted test protein intensity. The PAT/*pat* protein band was visible in the 0 and 60 minute incubation times without pepsin, with no decrease in stain intensity at 60 minutes. At time zero of incubation with SGF, the PAT/*pat* protein and the pepsin bands at approximately 36 kDa were clearly visible. The pepsin band at approximately 36 kDa showed no decrease in stain intensity at 60 minutes, indicating that the pepsin was stable.

Within 0.5 minutes and at all subsequent incubation times, the PAT/*pat* protein band was not visible on the SDS-PAGE gel and there were no bands visible at a lower molecular weight.

On the western blot conducted with the PAT/*pat* protein (Fig. 4), a major band located at approximately 21.5 kDa.was apparent. The binding of polyclonal anti-PAT/*pat* antibody further confirmed the identity of the PAT/*pat* protein. The 10% PAT/*pat* protein control was also visible with lower intensity compared to the undiluted test protein intensity.

At 0.5 minutes and all subsequent incubation times, the PAT/*pat* protein band was not visible on the western blot.

Therefore, the digestion pattern observed on the western blot was similar to the Coomassie blue stained SDS-PAGE, with a clear PAT/*pat* band at time zero, and a rapid digestion (>90%) from 0.5 minutes onward and no visible bands in the SGF alone lane.

Overall, more than 90% of PAT/*pat* protein was degraded within 0.5 minutes.

2 - HRP UNSTABLE REFERENCE PROTEIN (Fig. 2)

The HRP protein was visible at approximately 46 kDa in the 0 and 60 minute incubation times without pepsin, with no decrease in stain intensity at 60 minutes. The 10% HRP protein control was also visible with lower intensity. The pepsin at approximately 36 kDa band showed no decrease in stain intensity at 60 minutes, indicating that the pepsin was stable.
At time 0 of incubation with SGF, the HRP protein and the pepsin at approximately 36 kDa bands were clearly visible. At 30 seconds and subsequent incubation times, there was no HRP band at 46 kDa, and no smaller bands.

This indicates a complete digestion of the horseradish peroxidase unstable reference protein within 30 seconds.

3 - OVA STABLE REFERENCE PROTEIN (FIG. 3)

The OVA protein represented as two bands was clearly visible at approximately 45 kDa in the 0 and 60 minute incubation time without pepsin, with no decrease in stain intensity at 60 minutes. The 10% OVA protein control was also visible with lower intensity. The pepsin at approximately 36 kDa band showed no decrease in stain intensity at 60 minutes, indicating that the pepsin was stable.
At time 0 of incubation with SGF, the OVA protein bands (at approximately 45 kDa) and the pepsin (at approximately 36 kDa) bands were clearly visible. From 0.5 minutes up to 60 minutes incubations, the OVA protein bands were gradually reducing in intensity.

At 60 minutes, the OVA protein bands were still faintly visible, indicating that the OVA stable reference protein was not totally digested within 1 hour.

4 - DISCUSSION

The results showed that the pepsin was active, and that two reference proteins, HRP and OVA, were rapidly and slowly digested, respectively. The results of the reference proteins are in line with the results obtained in an international ring trial organized by ILSI (1). These quality control procedures confirm that the study procedures and reagents were adequate to detect the rate of digestion of proteins in this SGF study.

The PAT/*pat* protein was degraded very rapidly with less than 10% residual protein visible at 30 seconds of incubation with SGF, in presence of pepsin, at pH 1.2. There was also no significant digestion at pH 1.2 in the absence of pepsin, showing that digestion requires pepsin.

CONCLUSION

The PAT/*pat* protein was degraded very rapidly in human simulated gastric fluid, within 30 seconds of incubation, in presence of pepsin, at pH 1.2.

PROTOCOL DEVIATIONS

1- Addition of sucrose in SDS-PAGE samples:
In order to increase the viscosity of the SDS-PAGE samples, few grains of sucrose were added in the 5X Laemmli solution, which was used for running all SDS-PAGE samples.

2- Modification of the experimental starting date:
The experimental starting date was March 18, 2009, and not March 23 2009 as originally stated in the study protocol, as the reference proteins were weighted in March 18, 2009.

3- Preparation of the kaleidoscope molecular weight marker:
Prior to running, 10 µl of 5X Laemmli were added to 5 µl of kaleidoscope molecular weight marker.

It is the opinion of the Study Director that these deviations did not affect the results of the study.

Author / Study Director: [redacted]

Date: June 26, 2009

REFERENCES

DART Numbers

1 M-229898-01-1 THOMAS, K., AALBERS, M., BANNON, G.A., BARTELS, M, DEARMAN, R.J., ESDAILE, D.J., FU, T.J., GLATT, C.M., HADFIELD N, HATZOS C, HEFLE, S.L., HEYLINGS, J.R., GOODMAN, R.E., HENRY, B., HEROUET, C., HOLSAPPLE, M., LADICS, G.S., LANDRY, T.D., MACINTOSH, S.C., RICE, E.A., PRIVALLE, L.S., STEINER, H.Y., TESHIMA, R., VAN REE, R., WOOLHISER, M., ZAWODNY, J. (2004): A multi-laboratory evaluation of a common *in vitro* pepsin digestion assay protocol used in assessing the safety of novel proteins. *Regulatory Toxicology and pharmacology*, 39, pp. 87-98.

2 M-273056-01-1 UNITED STATES PHARMACOPEA (1990): Vol XXII, pp. 1788-1789, United States Pharmacopeia Convention, Inc, Rockville, MD.

3 M-223866-01-1 LAEMLLI U.K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970, 227, pp. 680-5.

4 M-273931-01-1 NEUHOFF V., AROLD N., TAUBE D., EHRHARDT W. (1988): Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis*, 9, pp. 255-62.

5 M-247779-01-1 HEROUET, C., ESDAILE, D.J., MALLYON, B.A., DEBRUYNE, E., SCHULTZ, A., CURRIER, T., HENDRICKX, K., VAN DER KLIS, R-J., ROUAN, D. 2005: Safety evaluation of the phosphinothricin acetyltransferase proteins encoded by the *pat* and *bar* sequences that confer tolerance to glufosinate-ammonium herbicide in transgenic plants. *Reg. Tox. Pharmacol*., 41, pp. 134-149.

ABBREVIATIONS

%	Percentage
°C	Degree (s) Celcius
µg	Microgram (s)
µl	Microliter (s)
DTT	Dithiothreitol
ECL	Enhanced ChemiLuminescence
EDTA	Ethylendiamineteraacetic acid
g	Gram (s)
g/ml	Gram/milliliter
GLP	Good Laboratory Practice
HRP	Horseradish Peroxidase
ILSI	International Life Science Institute
kDa	Kilodalton
kg	Kilogram (s)
M	Molar
mg	Milligram
mg/ml	Milligram/milliliter
Min	Minute (s)
ml	Milliliter
mm	Millimeter(s)
mM	Millimolar
NaCl	Sodium Chloride
OVA	Ovalbumin
PAGE	Polyacrylamide gel electrophoresis
PAT	Phosphinothricin acetyltransferase
PBS	Phosphate buffered saline
pH	Potential of hydrogen
SDS	Sodium dodecyl sulfate
SGF	Human simulated gastric fluid
Tris	Tris hydroxymethyl aminomethane
USA	United States of America
w/v	Weight/volume

FIGURES

Note: For clarity reasons, only the molecular weight maker 12 is presented in the following figures. The kaleidoscope molecular weight maker lane can be seen on the raw data file pictures.

Fig 1: Coomassie blue stained SDS-PAGE of PAT/*pat* protein after incubation in simulated human gastric fluid

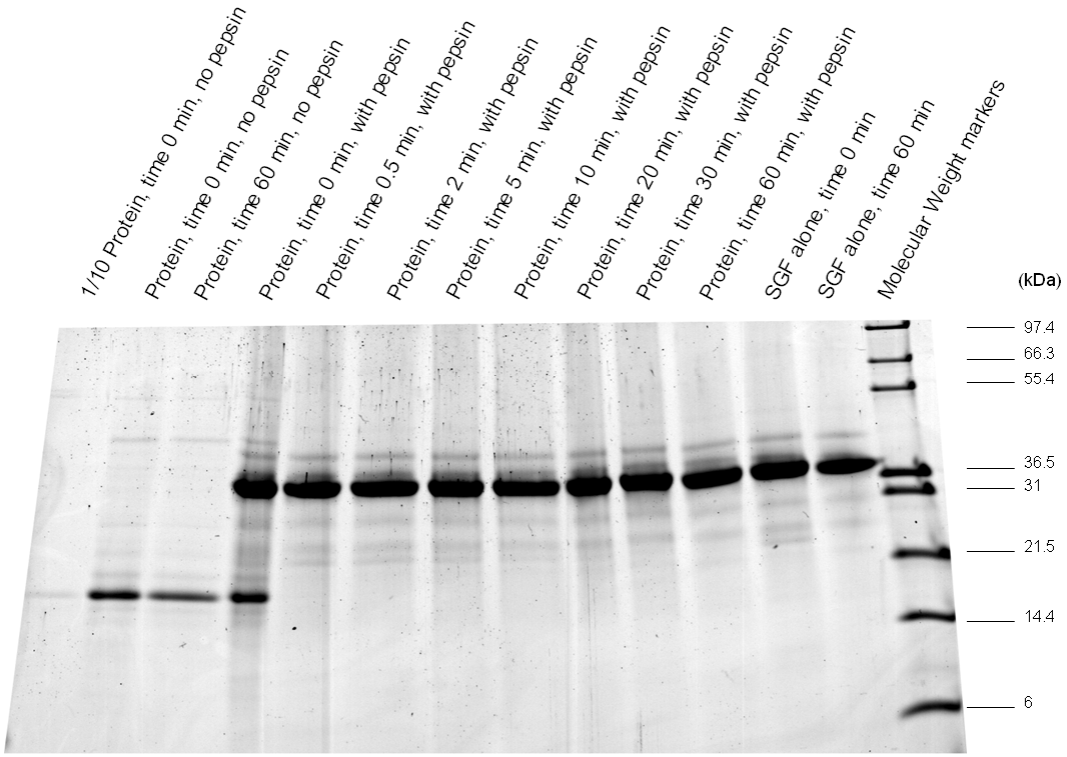


Fig 2: Coomassie blue stained SDS-PAGE of Horseradish Peroxidase (HRP) protein after incubation in simulated human gastric fluid

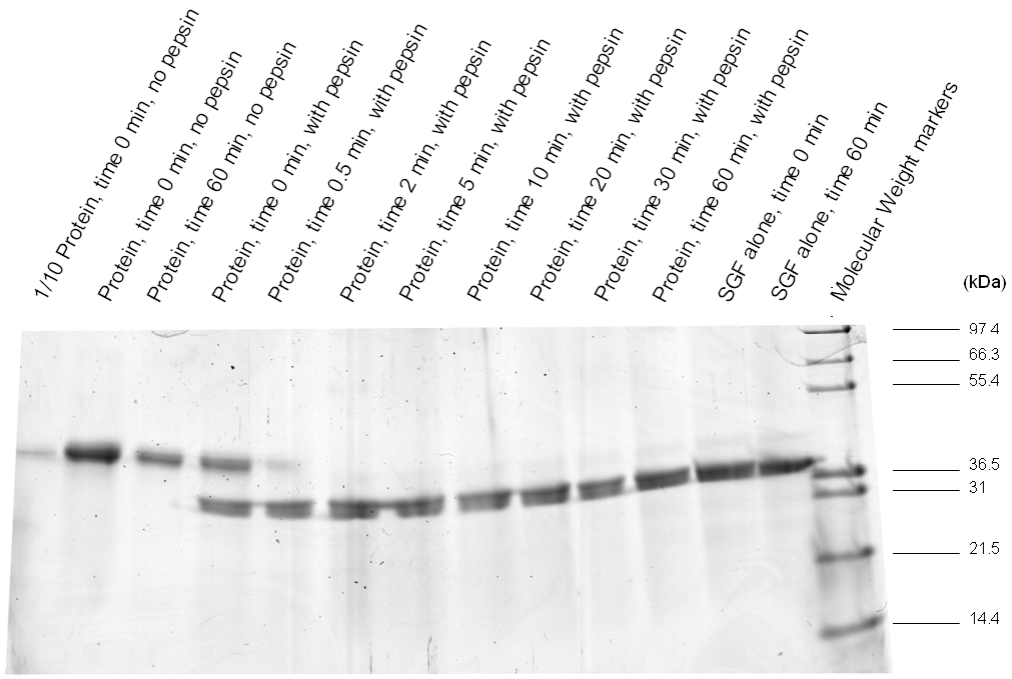


Fig 3: Coomassie blue stained SDS-PAGE of Ovalbumin (OVA) protein after incubation in simulated human gastric fluid

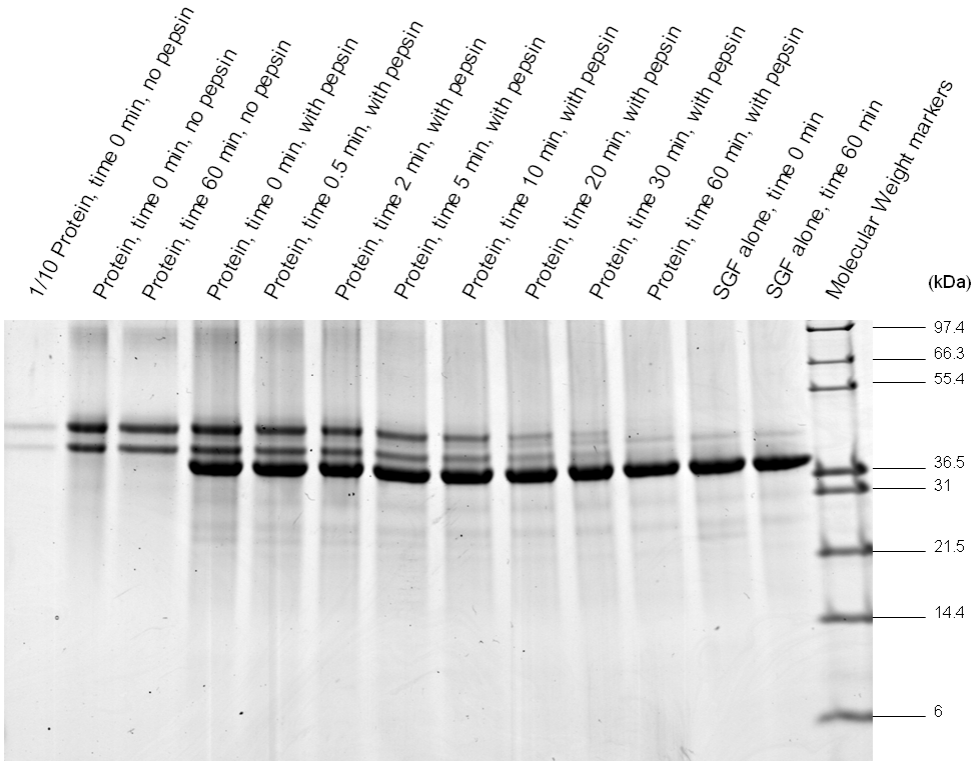
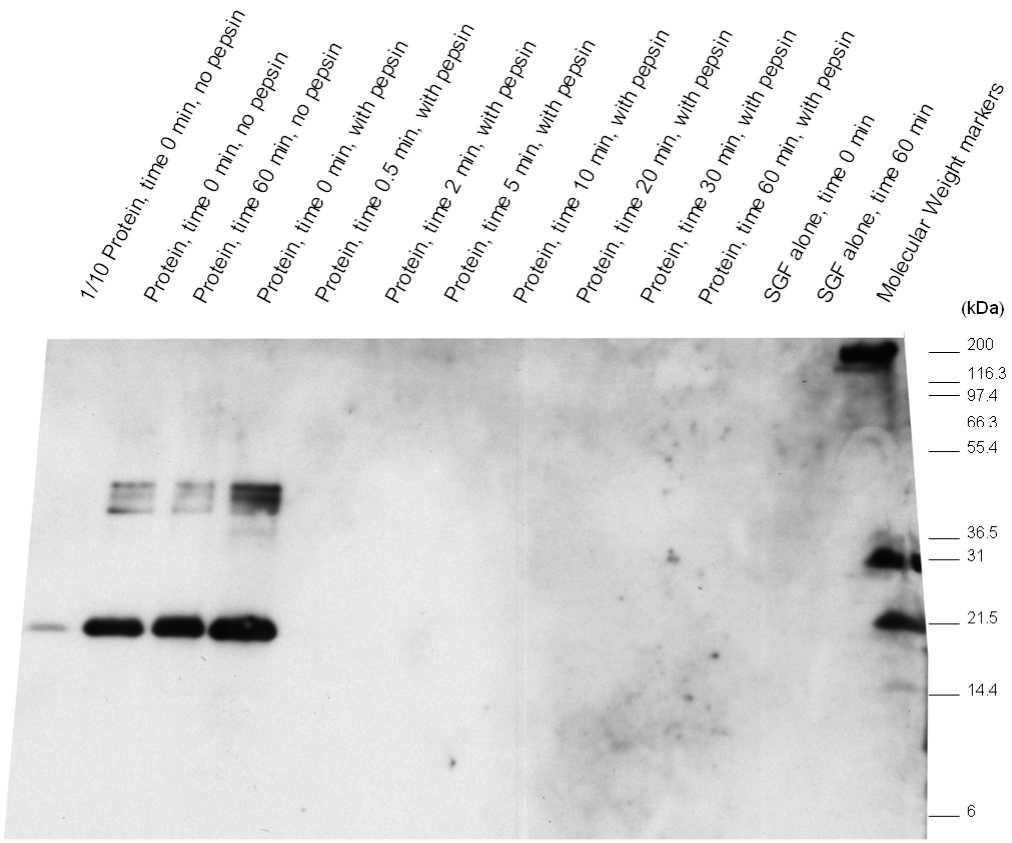


Fig 4: Western blot of PAT/*pat* protein after incubation in simulated human gastric fluid



ATTACHMENTS

ATTACHMENT 1 - **PROTOCOL**

PAT/*pat* PROTEIN
***IN VITRO* DIGESTIBILITY STUDY IN HUMAN SIMULATED GASTRIC FLUID**

TESTING FACILITY:
Bayer CropScience
355, rue Dostoïevski
BP 153
06903 Sophia Antipolis Cedex
France

SPONSOR:
Bayer AG
Bayer CropScience
Alfred Nobel Str. 50
40789 Monheim
Germany

1 GENERAL

1.1 PURPOSE OF STUDY

The present study is designated to determine the stability of the PAT protein, (encoded by the *pat* gene, produced in *E. coli*), in human simulated gastric fluid (SGF). The *in vitro* protein degradation will be observed in presence of pepsin at pH 1.2. The reference proteins horseradish peroxidase and ovalbumin will be tested concurrently as they are known to be respectively rapidly and slowly digested.

1.2 GOOD LABORATORY PRACTICE COMPLIANCE

This study will be performed in accordance with the principles of Good Laboratory Practice ("Bonnes Pratiques de Laboratoire") described in the following issues, with the exception of the test item solution, which will not be analyzed for concentration, homogeneity and stability.

- Organization for Economic Cooperation and Development (O.E.C.D.) Principles of Good Laboratory Practice, 1997 (January 26, 1998).
- European Directive 2004/10/EC (February 11, 2004).
- U.S. Environmental Protection Agency (E.P.A.)
  . 40 CFR Part 160
  Federal Insecticide, Fungicide and Rodenticide Act (FIFRA);
  Good Laboratory Practice Standards: Final Rule, August 17, 1989.
- Good Laboratory Practice Standards for Toxicology studies on Agricultural chemicals, Ministry of Agriculture, Forestry and Fisheries (M.A.F.F.) in Japan, notification 11 Nousan N°6283, October 01, 1999, modified by: 12 Nousan N°8628, December 06, 2000.
- French Decree N°2006-1523, regarding Good Laboratory Practice (December 04, 2006).

1.3 REGULATORY GUIDELINES

No data requirement for this study.

1.4 QUALITY ASSURANCE

The Quality Assurance Unit of Bayer CropScience, 355 rue Dostoïevski, BP 153, 06903 Sophia Antipolis Cedex, France, will undertake and document inspections while the study is in progress and will audit the study report.

2 STUDY PERSONNEL

2.1 STUDY DIRECTOR:

[redacted]

Date: March 13, 2009

2.2 SPONSOR REPRESENTATIVE:

[redacted]

Date: March 13, 2009

2.3 OTHER STUDY PERSONNEL

Responsibility		Name
Replacement Study Director	:	[redacted]
Responsible Technician	:	

Other study personnel will be identified as appropriate in the study file.

3 PROPOSED DATES

Experimental starting date	:	March 23, 2009	
Experimental completion date	:	April 03, 2009	(estimated)

4 OVERVIEW OF STUDY DESIGN

The method described in this protocol is based on Thomas *et al.* (2004) publication.

Test protein or reference protein solutions will be incubated with human simulated gastric fluids (SGF), (a pepsin solution at pH 1.2), at approximately 37°C and samples will be taken for analysis at time-points of 0, 0.5, 2, 5, 10, 20, 30 and 60 minutes. The resultant solution will be analyzed for the presence of the PAT/*pat* protein or potential stable protein fragments by Coomassie blue staining and by western blot analysis after SDS-PAGE. The immunodetection will be performed using a polyclonal antibody directed against the PAT/*pat* protein. Appropriate controls will include the test protein without pepsin at pH 1.2, SGF without the test protein, a 10% loading control with a 1/10 dilution of the test protein (to verify the sensitivity of protein staining procedure). Reference proteins known to be digested rapidly and slowly, respectively the horseradish peroxidase (HRP) and ovalbumin (OVA) will be tested in parallel.

5 MATERIALS AND METHODS

5.1 PROTEINS AND PEPSIN

The test item, PAT/*pat* protein, will be supplied by BioAnalytics (Bayer BioScience NV, Gent, Belgium).

Test item identification	: PAT/*pat* protein (encoded by the *pat* gene; produced in *E. coli*)
Batch number	: ADW040708
Purity	: 87.5 %
Storage	: Dry and dark at room temperature
Certified through	: Will be given in the study report

The certificate of analysis will be attached to the study report.

Reference items will be the "rapid digestion" reference protein HRP (reference P6782, Sigma, France) and the "slow digestion" reference protein OVA (reference A5503, Sigma, France).
The pepsin used will be from Sigma, reference P6887.

5.2 PROTEIN SOLUTIONS

The test protein will be received lyophilized and will be solubilized in Tris 20mM pH 7.5, 5mM EDTA at the final concentration of 2.5 mg PAT/*pat* protein/mL.

Each reference protein will be prepared as a stock solution of 2.5 mg/mL in the same buffer.

Once the test protein is solubilised, it may be stored frozen at -20°C or colder

Protein dissolution will be evaluated by visual inspection).

5.3 TEST SYSTEM

The human simulated gastric fluid will be prepared as follows:

- Preparation of 200 mL of G-con solution (2 mg/mL NaCl, pH 1.2).
- Preparation of the SGF solution: addition of the pepsin to the G-con solution. The quantity of pepsin in the SGF solution will be calculated so that the final concentration of pepsin in the digestion tubes will be of 10 activity units per µg of test protein.

5.4 5X LAEMMLI BUFFER

5X Laemmli buffer contains: 40% glycerol, 5% β-mercaptoethanol (BME), 10% SDS, 0.33 M Tris-HCl pH 6.8, 0.05% bromophenol blue.

5.5 DIGESTION

A dilution of the test protein solution at 1/10 in Tris 20mM pH 7.5, 5mM EDTA will be prepared for the 10% loading control.

The microcentrifuge tube of SGF for the serial digestion samples, and the control tubes (G-con + protein time 60 min and SGF alone, 60 min) will be pre-warmed in a 37°C waterbath for approximately 2 minutes before the time zero (prior to the addition of protein / H_2O MilliQ).

The "serial digestion" tube prepared for the test protein digestion will contain 1520 µl of the SGF for sampling from 0.5 to 60 minutes.

Eighty µl of protein solution will be added (time zero) and briefly agitated then returned to the waterbath at 37°C.

At 0.5, 2, 5, 10, 20, 30 and 60 minutes, samples of 200 µl will be taken from tube; prior to each sampling, tubes will be briefly agitated. An additional agitation at approximately 45 minutes without sampling will be performed.

Control tubes:

G-con + protein 1/10 time 0	190 µl G-con (SGF without pepsin) + 10 µl protein diluted to 1/10 at time zero
G-con + protein time 0	190 µl G-con (SGF without pepsin) + 10 µl protein: sample at time zero
G-con + protein time 60 min	190 µl G-con (SGF without pepsin) + 10 µl protein: sample at 60 minutes (vortex and incubate in waterbath at 37°C)
SGF + protein time 0	190 µl SGF + 10 µl protein (added last after mixing): sample at time zero
SGF alone, 0 min	190 µl SGF + 10 µl H_2O MilliQ: sample at time zero
SGF alone, 60 min	190 µl SGF + 10 µl H_2O MilliQ: sample at 60 minutes (vortex and incubate in waterbath at 37°C)

The same procedure will be followed for the two reference proteins.

5.6 SAMPLES

The reaction will be stopped as soon as samples are taken.

For the control tubes (G-con + protein time 60 min and SGF alone, 60 min), each containing 200 µl of sample, the neutralisation solution (70 µl 200 mM NaHCO3 pH 11.0) will be added directly to the incubation tubes.

For the serial digestion samples and the other control tubes, the reaction will be terminated by adding the 200 µl of digestion sample to a tube containing 70 µl of 200 mM NaHCO3 (pH 11.0).

Samples may be directly used for analysis on SDS-PAGE gels or frozen at -20°C.

5.7 SDS-PAGE ANALYSIS

The method will be based on Laemmli's method (1970) using a Mini-Protean III cell (BioRad, France). Prior to running SDS-PAGE, 5 µl of 5X Laemmli solution will be added to 20 µl of digestion samples and heated for 10 minutes at >90°C before loading the gel. Samples of 15 µl will be added to wells of an SDS-PAGE gel (15 wells, 1 mm 10-20% gradient polyacrylamide Tris-Tricine, BioRad).

Suitable molecular weight markers will be used to provide reference points of known molecular weights on the gel (Kaleidoscope, prestained standard, Biorad and Mark 12, Invitrogen, France). Prior to running, 10 µl of 5X Laemmli will be added to 5 µl of Marker 12.

For each test or reference protein "serial digestion" and control samples will be loaded on the same gel.

Loading order on each gel:

Lane	Sample
1	Kaleidoscope
2	G-con + protein 1/10 time 0
3	G-con + protein time 0
4	G-con + protein time 60 min
5	SGF + protein time 0
6	SGF + protein time 0.5 min
7	SGF + protein time 2 min
8	SGF + protein time 5 min
9	SGF + protein time 10 min
10	SGF + protein time 20 min
11	SGF + protein time 30 min
12	SGF + protein time 60 min
13	SGF alone, 0 min
14	SGF alone, 60 min
15	Marker 12

Electrophoresis will be carried out with a constant voltage set at 100 volts until the bromophenol line has reached the bottom of the gel.

Gels will be stained by the Coomassie blue method (Colloidal blue staining kit, Invitrogen, USA) and the gels will be scanned. The gels are not stable for more than a week, so the raw data will be the scanned image.

In addition, a second SDS-PAGE will be performed for the western blot analysis.

This gel will be loaded with approximately 200 times less protein in quantity to take into account the better level of detection of the western-blot technique compared to one of Coomassie blue staining.

5.8 WESTERN BLOT ANALYSIS

A polyvinylidene fluoride (PVDF) membrane will be placed on the gel in a Tris/Glycine buffer and an electrical current applied in order to transfer the protein bands onto the membrane. To detect the PAT/*pat* protein bands and/or its potential fragments recognized by a specific anti-PAT/*pat* protein antibody, the membrane will be incubated in the presence of a specific rabbit polyclonal anti-PAT/*pat* protein antibody.

The hybridization of the antibody with the proteins immobilized on the membrane will be revealed using a goat anti-rabbit polyclonal antibody coupled with a Horseradish Peroxydase. The hybridization bands will be visualized using chemoluminescent (ECL) detection system (Amersham, France).

The autoradiographs will be scanned (Biorad) and resulting image will be retained in the raw data and reported in the final report.

5.9 DATA ANALYSIS

The results of a detailed visual inspection of the scans will be reported.

The gel will be considered to be valid if:

- the pepsin band is at the correct molecular weight (approximately 36 kDa) and is stable throughout the study.
- the protein band is clearly visible at time zero (lanes 2 and 3) and is not visible in the lanes without protein (lanes 13 and 14).
- at least one marker lane is clearly visible to allow a molecular weight evaluation.

The study will be considered valid if the gels are valid and the reference proteins give results that are in line with expected data (i.e., rapid digestion for HRP and slow digestion for OVA).

The time at which the parent protein disappears will be reported.

If stable fragments of the test protein are visible on the gel, then their number and time-course will be reported.

6 REPORTING

A copy of the draft report will be submitted to the Sponsor Representative and the Quality Assurance Unit for review. With the exception of the dated signature of scientists and other professional personnel, the draft report will contain all information and data to be included in the final report. The final report will include the information and data required by current internationally recognized regulations.

7 ARCHIVING

All raw data, supporting documents as well as protocol, protocol amendments and final report will be maintained in the archive room. An aliquot of the test substance solution will be kept in the area of the products storeroom defined for the archiving of test substances.

All of the above will be archived for at least 10 years in the designated areas at:

Bayer CropScience
355, rue Dostoïevski
BP 153
06903 Sophia Antipolis Cedex
France

8 REFERENCES

THOMAS *et al.*, (2004): A multi-laboratory evaluation of a common *in vitro* pepsin digestion assay protocol used in assessing the safety of novel proteins. *Regulatory Toxicology and Pharmacology*, 39, pp. 87-98.

LAEMMLI, U.K., (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 1970, 227, pp. 680-5.

ATTACHMENT 2 - **CERTIFICATES OF ANALYSIS**

Bayer CropScience

BioScience

CERTIFICATE OF ANALYSIS BA-02-ADW

Origin of the Certified Material
Bayer BioScience N.V.
Bioanalytics
Protein Characterization
Technologiepark 38
B-9052 Zwijnaarde, Belgium
Tel : +32 (0)9 243 0411
Fax : +32 (0)9 224 0694

Bayer BioScience N.V.
Bioanalytics
Technologiepark 38
B-9052 Zwijnaarde
Belgium

Date : 11-09-2008

General Protein Information

- Product name : lyophilized PATpat protein of batch # NB170206P109
- Batch number : **# ADW040708**
- Produced by : Bayer Bioscience NV
- Amount : aliquoted in 0.5 mg protein (OD280)
- Storage : dry and dark at roomtemperature

- Solubility : - not soluble in PEG-buffer: 70% PEG400; 20 % DMF; 10 % water
  - Soluble in Tris pH 7.5 + 1 mM DTT till 1 mg/ml
  - Soluble in PBS + 1 mM DTT till 1 mg/ml
  - Soluble in PB buffer without DTT till 1 mg/ml

Analyses performed on a 1mg/ml solution in Tris buffer(1) or Phosphate buffer without salts (2) :

- protein concentration : Resolubilisation till 1 mg/ml (1) gives a 100 % recovery. After resolution, the protein concentration has to be determined by measuring the OD280 (extinction coefficient 44500, 1 OD280 corresponds to 0.46 mg/ml)
- purity: 87.5 %, determined by means of SDS-PAGE (NuPage 10 % in MES buffer) and analysed using the G-box (SynGene) (attachment 1).
- immunodetection: Immunodetection OK. Testing method:
  - A dilution serie of 100 ng till 0.5 ng of the pure PATpat protein #NB170206P109 and of the lyophylized PATpat protein #ADW040708 (2) was spotted on dot blot
  - blocking in PBS/Tween 20 0.2 %
  - Immunodetection with rabbit anti PATpat #N.100608 (1/1000)
  - secundary antibody A8025 goat anti rabbit –AP (1/7000)
  - NBT/BCIP detection

  → detectionlimit for NB170206P109 = 3.1 ng and for ADW040708 = 6.2 ng

  This lyophilized protein can not be used as standard in ELISA (attachment 2)
- activity: Active after dialyzing and lyophilizing and dissolving in Tris buffer. (SOP BBS 07/72/00)
  The components of the PEG-buffer showed no interference in the activity assay.
- Stability : not known yet
  To be used immediately after resolution.
- Attachments : 2

[redacted] Lableader of BA - protein

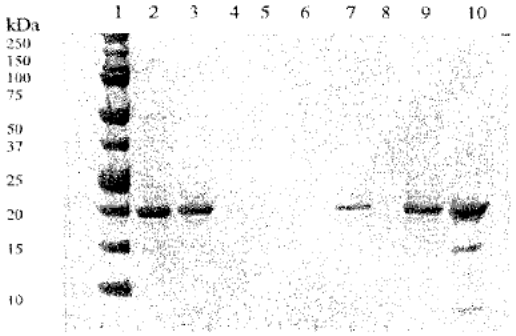
12/9/09

[redacted]

Bayer CropScience

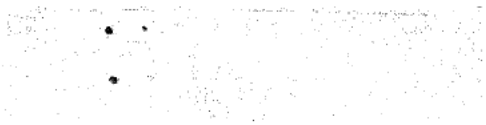
BioScience

Attachment 1: SDS-PAGE Nupage 10 % in MES buffer ; *10 µl loaded*



1 : BioRad Precision Plus Protein standard #161-0374
2 : # NB170206p109 6 µg
3 : dialyzed NB170206p109 in $(NH_4)_2CO_3$: 5 µg
4: dissolved ADW040708 5 mg/ml in PEG buffer, expected amount 50 µg
5: undissolved ADW040708 5mg/ml in PEG buffer
6: not loaded
7: undissolved ADW040708 5 mg/ml in Tris buffer
8: dissolved ADW040708 in PEG buffer; expected amount 10 µg
9: dissolved ADW040708 1mg/ml in Tris buffer, expected amount 10 µg
10: dissolved ADW040708 5mg/ml in Tris buffer, expected amount 50 µg

Attachment 2: immuno dot blot on the pure protein #NB170206P109 and the lyophilized form #ADW040708 with rabbit anti PATpat #N.100608 (1/1000):
Spots of 100 ng, 50, 25, 12.5, 6, 3, 1.5, 0.75



NB170206P109: detectionlimit 3 ng

ADW040708: detectionlimit 6 ng

Certificate of Analysis

SIGMA-ALDRICH

Product Name	Peroxidase from horseradish, Type VI-A, essentially salt-free, lyophilized powder, ~1000 units/mg solid (using ABTS), 250-330 units/mg solid (using pyrogallol)
Product Number	P6782
Product Brand	SIGMA
CAS Number	9003-99-0

TEST	SPECIFICATION	LOT 125K7415 RESULTS
APPEARANCE	RED-BROWN POWDER	CONFORMS
SOLUBILITY	CLEAR AMBER TO AMBER-BROWN SOLUTION AT 10 MG PLUS 1 ML OF 0.1 M PHOSPHATE BUFFER, PH 6.0	CLEAR AMBER-BROWN
ENZYMATIC ACTIVITY	APPROX. 1000 UNITS PER MG SOLID (USING ABTS)	1,080 UNITS/MG SOLID
UNIT DEFINITION	ONE UNIT WILL OXIDIZE 1 MICROMOLE OF 2,2'-AZINO-BIS(3-ETHYLBENZTHIAZOLINE-6-SULFONIC ACID) PER MINUTE AT 25 DEG C AT PH 5.0.	
ENZYMATIC ACTIVITY	250 TO 330 UNITS/MG SOLID (USING PYROGALLOL)	298 UNITS/MG SOLID
UNIT DEFINITION	ONE UNIT WILL FORM 1.0 MG OF PURPUROGALLIN FROM PYROGALLOL IN 20 SECONDS AT PH 6.0 AT 20 DEG C.	
RZ	APPROX. 3.0. THE RZ IS DEFINED AS THE RATIO OF THE ABSORBANCE AT 403NM TO THE ABSORBANCE AT 275NM. THIS VALUE IS AN EXPRESSION OF THE RATIO OF HEMIN TO PROTEIN CONTENT.	RZ: 2.9
RECOMMENDED RETEST	5 YEARS	FEBRUARY 2011
QC RELEASE DATE		FEBRUARY 2006

[redacted]
Analytical Services
St. Louis, Missouri USA

Certificate of Analysis

SIGMA-ALDRICH

Product Name	Albumin from chicken egg white, Grade V, ≥ 98% (agarose gel electrophoresis), lyophilized powder
Product Number	A5503
Product Brand	SIGMA
CAS Number	9006-59-1

TEST	SPECIFICATION	LOT 126K7009 RESULTS
APPEARANCE	WHITE TO YELLOW POWDER	WHITE POWDER
SOLUBILITY	CLEAR TO SLIGHTLY HAZY COLORLESS TO YELLOW SOLUTION AT 40MG/ML IN WATER	CLEAR FAINT YELLOW
WATER BY KARL FISCHER	NMT 6%	5%
ELEMENTAL ANALYSIS	13.0 TO 16.0% NITROGEN	14.7%
AGAROSE ELECTROPHORESIS	MINIMUM 98%	>99%
RECOMMENDED RETEST	5 YEARS	MARCH 2012
QC RELEASE DATE		MARCH 2007

[redacted]

Analytical Services
St. Louis, Missouri USA

Certificate of Analysis

SIGMA-ALDRICH

Product Name	Pepsin from porcine gastric mucosa, lyophilized powder, 3,200-4,500 units/mg protein
Product Number	P6887
Product Brand	SIGMA
CAS Number	9001-75-6

TEST	SPECIFICATION	LOT 056K7676Z2 RESULTS
APPEARANCE	REPORT RESULT	WHITE LYOPHILIZED POWDER
PROTEIN BY UV ABSORBANCE	REPORT RESULT	92%
ENZYMATIC ACTIVITY	3,200 TO 4,500 UNITS/MG PROTEIN	3,260 UNITS/MG PROTEIN
UNIT DEFINITION	ONE UNIT WILL PRODUCE A CHANGE IN A280 OF 0.001 PER MINUTE AT PH2.0 AT 37DEGC, MEASURED AS TCA-SOLUBLE PRODUCTS USING HEMOGLOBIN AS SUBSTRATE. (FINAL VOLUME = 16ML. LIGHT PATH = 1CM.)	
RECOMMENDED RETEST	2 YEARS	JUNE 2009
QC RELEASE DATE		JUNE 2008

[redacted]
Analytical Services
St. Louis, Missouri USA

FINAL REPORT AMENDMENT

There is no final report amendment at this time.

This page has been left blank intentionally.