Bayer CropScience

PAT/*pat* protein

IN VITRO DIGESTIBILITY STUDY IN

HUMAN SIMULATED INTESTINAL FLUID

DATA REQUIREMENT
No applicable guidelines

REPORT OF STUDY SA 09049
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 24, 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 24, 2009

Sponsor Representative: [redacted]

Date: June 24, 2009

Study Submitter:

Date:

FLAGGING STATEMENTS

This page is reserved for flagging statements as may be required by US EPA.

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	Digestion	March 16, 2009	March 16, 2009	March 27, 2009
	Migration	March 18, 2009	March 18, 2009	March 27, 2009
Process-based	Western blot	April 02, 2009	April 06, 2009	April 08, 2009
Study-based	Final report	June 17, 2009	June 17, 2009	June 22, 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 24, 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 24, 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]

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SUMMARY

The PAT/*pat* protein (encoded by the *pat* gene**,** produced in *Escherichia coli*) was tested for stability in human simulated intestinal fluid (SIF) with pancreatin at pH 7.5 for incubation times from 0.5 to 60 minutes. The protocol was based on the International Life Science Institute (ILSI) protocol using simulated gastric fluid (1). This protocol was adapted to use human Simulated Intestinal Fluids (SIF).

A solution of the test protein was incubated with simulated intestinal fluid (SIF) (a porcine pancreatin solution at pH 7.5) at approximately 37 °C and samples were taken for analysis at time-points of 0, 0.5, 2, 5, 10, 20, 30 and 60 minutes. The resultant solutions were analyzed for the presence of the PAT/*pat* protein or potential stable protein fragments by western blot analysis. The immunodetection was performed using a polyclonal antibody directed against PAT/*pat* protein. Appropriate controls included PAT/*pat* protein in buffer without pancreatin, the corresponding 10% loading condition (to verify the sensitivity of the detection procedure) and SIF without PAT/*pat* protein.

The PAT/*pat* protein (encoded by the *pat* gene, produced in *Escherichia coli*) was degraded very rapidly, within 5 minutes of incubation. At 5 minutes of incubation, less than 10% of the PAT/*pat* protein remained. The PAT/*pat* protein was completely degraded within 10 minutes of incubation with SIF, in presence of pancreatin, at pH 7.5.

INTRODUCTION

This *in vitro* human simulated intestinal fluid (SIF) digestibility study has been conducted to provide a full GLP study carried out in line with a current internationally recognized protocol. This study is based on the protocol of the methods used in the 2004 ILSI ring trial (1). The method is based on the United States *Pharmacopea* (2). This protocol was adapted to use human Simulated Intestinal Fluids (SIF).

In this study the test material was the PAT/*pat* protein. (encoded by the *pat* gene, produced in *E. coli*).

The study protocol and amendment are 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 24, 2009
Experimental completion date	June 15, 2009

* Date of protocol approval by Study Director

MATERIAL AND METHODS

1 - PROTEINS AND PANCREATIN

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

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

The pancreatin protein was purchased from Sigma (P3292; Sigma, France).

Identification ..... :	Pancreatin (from porcine pancreas)
Appearance..... :	Tan powder
Concentration and purity.. :	Biological mixture of enzymes, concentration and purity not supplied by Sigma
Storage ..... :	Approximately -20°C
Certified through ..... :	Stable under storage conditions

The reference item azoalbumin (AZA) was purchased from Sigma (A2382).

Identification ..... :	Azoalbumin
Appearance..... :	Orange powder with a red cast
Purity ..... :	78%
Storage ..... :	Approximately 4°C
Certified through ..... :	April 2011

The certificates of analysis are presented in Attachment 2.

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.

3 - TEST SYSTEM

The SIF was prepared as follows:

- Preparation of I-con solution (6.8 g/l KH2PO4; titrate to pH 7.5 using NaOH)
- Addition of pancreatin (1% w/v) to the I-con solution.

In order to validate the activity of the pancreatin solution, a quality control procedure was carried out to demonstrate the degradation of the azoalbumin. The pancreatin solution was found to have a normal level of activity.

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 SIF and mixed. Samples of 200 µl were taken at 0.5, 2, 5, 10, 20, 30 and 60 minutes. The tubes were agitated after 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.

As soon as samples were taken, the reaction was terminated by adding the 200 µl sample to a tube containing 70 µl miliQ water placed on ice.

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

An aliquot of 20 µl of the samples was used for SDS-PAGE and the remaining was frozen at -20°C.

5 - SDS-PAGE ANALYSIS

The method was based on the method of Laemmli (3) using a Mini-Protean III cell (Bio-Rad, France). Prior to running SDS-PAGE, 5 µl of 5X Laemmli solution (possibly supplemented with sucrose) was added to 20 µl of digestion samples and heated for 10 minutes at >90°C before loading the gel. Samples of 15 µl were added to wells of an SDS-PAGE gel (15 wells, 1 mm 10-20% gradient polyacrylamide Tris-Tricine, Bio-Rad).

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

Gels were stained by the Coomassie blue method (Invitrogen) based on the work of Neuhoff *et al.* (5). After rinsing, the gels were scanned using a GS800 scanner (Biorad). The gels are not stable for more than two weeks, so the raw data were the scanned image.

In addition, a second SDS-PAGE was be 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/*pat* protein band and/or its potential fragments, the membrane was 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 was revealed using a goat anti-rabbit polyclonal antibody coupled with a peroxidase. The hybridization bands were visualized using enhanced chemoluminescence (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, protocol amendments, 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

1 - SDS-PAGE ANALYSIS (Fig. 1)

The SDS-PAGE analysis of the PAT/*pat* protein showed one major band located below the molecular weight marker at 21.5 kDa, as expected (5). The 10% PAT/*pat* protein control was also visible with lower intensity than the undiluted PAT/*pat* protein.
The PAT/*pat* protein band was visible in the zero and 60 minute incubation times without pancreatin, with no decrease in stain intensity at 60 minutes.
At time zero of incubation with SIF, the various pancreatin bands were clearly visible.
At time zero and all subsequent incubation times, the PAT/*pat* protein band was not visible on the gel, suggesting that either the pancreatin bands background was too high for allowing the observation of the PAT/*pat* protein band or that the PAT/*pat* protein was immediately digested.

2 - WESTERN BLOT ANALYSIS (Fig. 2)

The western blot analysis of the PAT/*pat* protein revealed a major band located below the molecular weight marker at 21.5 kDa, as expected (5). The 10% PAT/*pat* protein control was also visible with lower intensity than the undiluted PAT/*pat* protein. The binding of polyclonal anti-PAT/*pat* antibody further confirmed the identity of the PAT/*pat* protein.

Up to 5 minutes of incubation, the intact PAT/*pat* protein band was clearly visible, with a clear decreasing intensity from 2 minutes onward. At 5 minutes of incubation, the intensity of the PAT/*pat* protein was slightly below the intensity of the 10% PAT/*pat* protein control.

These results indicate that the PAT/*pat* protein was degraded very rapidly, within 5 minutes of incubation. At 5 minutes of incubation, less than 10% of the PAT/*pat* protein remained. The PAT/*pat* protein was completely degraded within 10 minutes of incubation.with SIF, in presence of pancreatin, at pH 7.5.

CONCLUSION

The PAT/*pat* protein (encoded by the *pat* gene, produced in *Escherichia coli*) was degraded very rapidly into fragments visible up to 5 minutes of incubation. The PAT/*pat* protein was completely degraded within 10 minutes of incubation with SIF, in presence of pancreatin, at pH 7.5.

PROTOCOL DEVIATION

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.

It is considered that this deviation did not affect the integrity of the study.

Author / Study Director:

[redacted]

Date: June 24, 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. 680-685.

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. 255-262.

5 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 Celsius
µg	Microgram(s)
µl	Microliter(s)
AZA	Azoalbumin
ECL	Enhanced ChemiLuminescence
EDTA	Ethylen-diamine tetra acetic acid
g	Gram(s)
g/l	Gram(s)/liter(s)
g/ml	Gram (s)/milliliter(s)
GLP	Good Laboratory Practice
H_2O MilliQ	Ultrapure water
ILSI	International Life Science Institute
kDa	Kilodalton(s)
kg	Kilogram(s)
KH_2PO4	Potassium phosphate monobasic
M	Molar
mg	Milligram(s)
mg/ml	Milligram(s)/milliliter(s)
min	Minutes(s)
ml	Milliliter(s)
mm	Millimeter(s)
mM	Millimolar
MW	Molecular Weight
NaOH	Sodium hydroxyde
PAT	Phosphinothricin acetyltransferase
pH	Potential of hydrogen
PVDF	Polyvinylidene Difluoride
SDS-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
SIF	Human simulated intestinal fluid
USA	United States of America
w/v	Weight/volume
Tris	Tris hydroxymethyl aminomethane

FIGURES

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

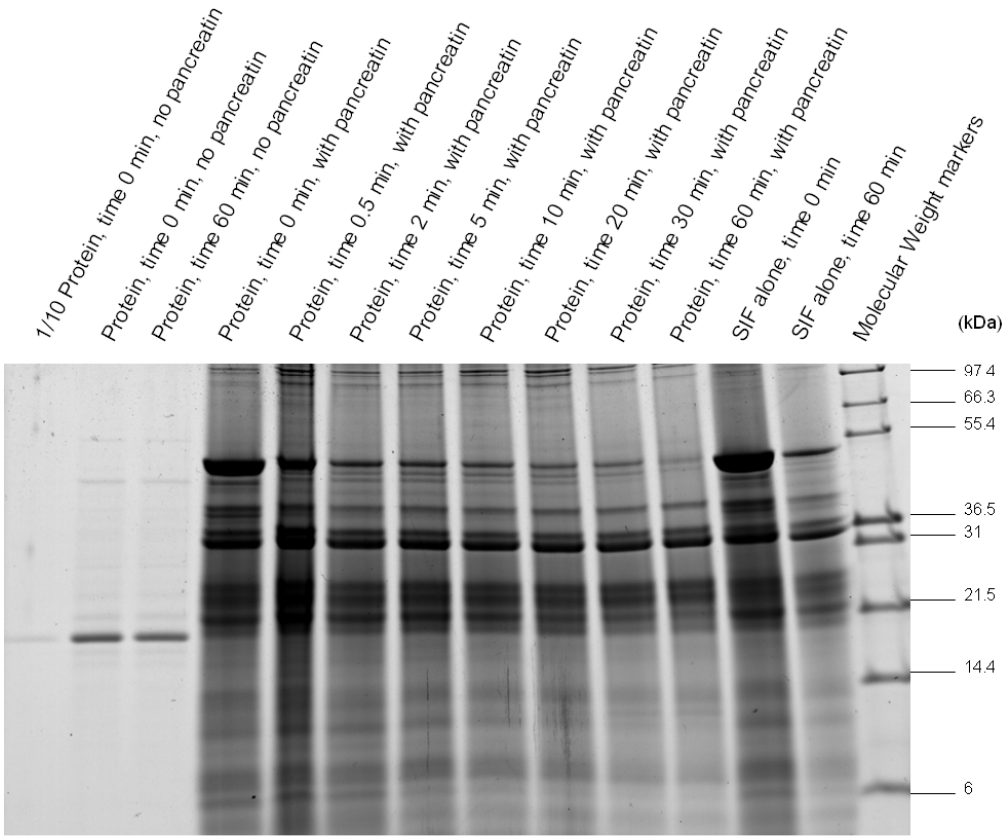
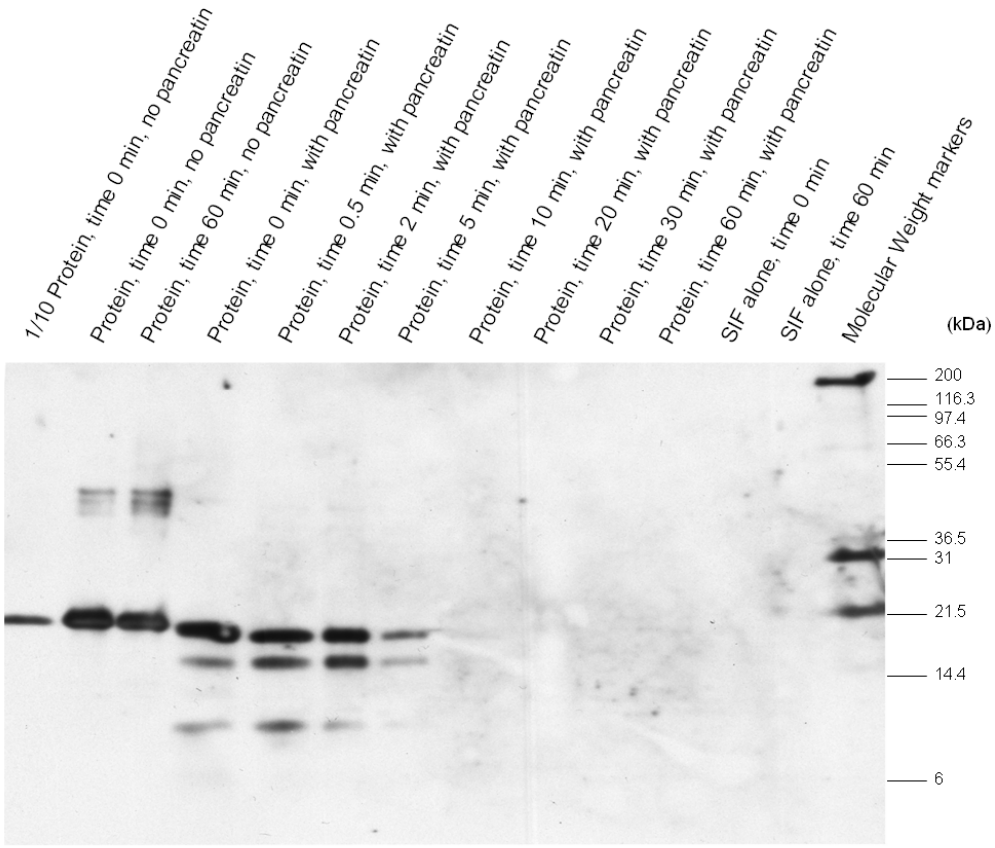


Fig 2: Western blot of PAT/*pat* protein after incubation in human simulated intestinal fluid



ATTACHMENTS

ATTACHMENT 1 - **PROTOCOL AND AMENDMENT**

PAT/*pat* PROTEIN:
***IN VITRO* DIGESTIBILITY STUDY IN HUMAN SIMULATED INTESTINAL 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*), by investigating the digestion of the protein using human simulated intestinal fluid (SIF). The *in vitro* protein degradation will be observed by Coomassie blue-stained SDS-PAGE and western-blot analyses and will give an indication of the potential stability of the protein in the digestive tract.

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 24, 2009	
Experimental completion date	:	April 03, 2009	(estimated)

4 OVERVIEW OF STUDY DESIGN

A solution of the test protein will be incubated with simulated intestinal fluid (SIF) (a porcine pancreatin solution at pH 7.5) 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 stained SDS-PAGE and by western blot analysis. The immunodetection will be performed using a polyclonal antibody directed against the PAT/*pat* protein. Appropriate controls will include PAT/*pat* protein in buffer without pancreatin, the corresponding 10% loading condition (to verify the sensitivity of the detection procedure) and SIF without PAT/*pat* protein.

5 MATERIALS AND METHODS

5.1 PROTEINS AND PANCREATIN

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.

The reference item (to show pancreatin activity) will be azoalbumin (A-2382, Sigma, France).

The pancreatin from porcine pancreas will be purchased from Sigma (P3292).

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 2.5mg PAT/*pat* protein/ml.

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

Protein dissolution will be evaluated by visual inspection.

5.3 TEST SYSTEM

The SIF will be prepared as follows:

- Preparation of 100 ml of I-con solution (6.8 g/l KH_2PO_4; titrate to pH7.5 using NaOH)
- Preparation of the SIF solution by addition of pancreatin (1% w/v) to the I-con solution.

To ensure that the pancreatin is active, it will be tested against a pancreatin substrate, azoalbumin, using a spectrophotometric endpoint. This test must show that the pancreatin is able to degrade azoalbumin rapidly.

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 (I-con + protein time 60 min and SIF 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 SIF 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:

I-con + protein 1/10 time 0	190 µl I-con (SIF without pancreatin) + 10 µl protein diluted to 1/10 at time zero
I-con + protein time 0	190 µl I-con (SIF without pancreatin) + 10 µl protein : sample at time zero
I-con + protein time 60 min	190 µl I-con (SIF without pancreatin) + 10 µl protein : sample at 60 minutes (agitate and incubate in waterbath at 37°C)
SIF + protein time 0	190 µl SIF + 10 µl protein (added last after mixing and cooling on ice)
SIF alone, 0 min	190 µl SIF + 10 µl H_2O Milli Q: sample at time zero
SIF alone, 60 min	190 µl SIF + 10 µl H_2O Milli Q: sample at 60 minutes (agitate and incubate in waterbath at 37°C)

5.6 SAMPLES

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

For the control tubes (I-con + protein time 60 min and SIF alone, 60 min), each containing 200 µl of sample, the neutralisation solution (70 µl H_2O MilliQ) 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 H_2O Milli Q and placed on ice.

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

5.7 SDS-PAGE ANALYSIS

The method will be based on that of Laemmli (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	Markers (kaleidoscope)
2	I-con + protein 1/10 time 0
3	I-con + protein time 0
4	I-con + protein time 60 min
5	SIF + protein time 0
6	SIF + protein time 0.5 min
7	SIF + protein time 2 min
8	SIF + protein time 5 min
9	SIF + protein time 10 min
10	SIF + protein time 20 min
11	SIF + protein time 30 min
12	SIF + protein time 60 min
13	SIF alone, 0 min
14	SIF alone, 60 min
15	Markers (Mark 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 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/*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 scanner) 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 pancreatin bands are generally stable throughout the study on the gel.
- the protein or fragment bands are clearly visible at time zero (lanes 2 and 3) and not visible in the lanes without protein (lanes 13 and 14).
- at least one marker lane is clearly visible on the SDS gel to allow a molecular weight evaluation.

The study will be considered valid if the gels are valid.

The Commassie blue-stained gel and the autoradiograph will be digitalized using high-resolution scanner (Biorad).

The time at which the test protein disappears will be reported. If stable fragments of the test protein are visible on the gels or on the blot, 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 REFERENCE

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

PROTOCOL AMENDMENT

Protocol **SA 09049**

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

Protocol amendment: N°1

Reason 1: Additional information concerning the spectrophotometric detection software

The version of the spectrophotometric detection software used for the pancreatin detection is Cary WinUV Version 3.0 (182).

Reason 2: Typing error in section 5.5 (Digestion) of the study protocol

The digestion sample should read as SIF instead of SGF, as originally stated in section 5.5 of the study protocol.

Study Director: [redacted]

Date: March 27, 2009

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

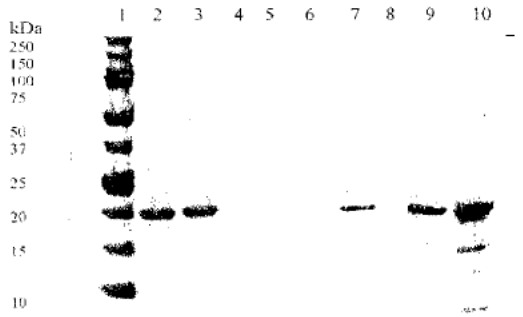
Responsible Scientist and Lableader of BA - protein

[redacted] 12/9/08

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

Certificate of Analysis

SIGMA-ALDRICH

Product Name	Pancreatin from porcine pancreas, powder, 4 × USP specifications, cell culture tested
Product Number	P3292
Product Brand	SIGMA
CAS Number	8049-47-6

TEST	SPECIFICATION	LOT 117K1343 RESULTS
APPEARANCE	LIGHT YELLOW TO TAN POWDER	TAN POWDER
DIGESTIVE POWER	4X USP SPECIFICATIONS	CONFORMS*
CELL CULTURE TEST	PASS	PASS
		* SUPPLIER'S INFORMATION
QC RELEASE DATE		DECEMBER 2007

[redacted]
Analytical Services
St. Louis, Missouri USA

Certificate of Analysis

SIGMA-ALDRICH

Product Name	Azoalbumin
Product Number	A2382
Product Brand	SIGMA
CAS Number	102110-73-6

TEST	SPECIFICATION	LOT 026K7565 RESULTS
APPEARANCE	ORANGE TO ORANGE-BROWN WITH A RED CAST POWDER	ORANGE POWDER WITH A RED CAST
SOLUBILITY	RED-ORANGE TO RED-BROWN SOLUTION AT 25MG/ML IN 0.01N HYDROCHLORIC ACID	RED-ORANGE SOLUTION
PROTEIN BY LOWRY	REPORT RESULT	78%
SUITABILITY	SUITABLE FOR USE AS A TRYPSIN SUBSTRATE	CONFORMS
UV-VIS SPECTRUM	LAMBDA MAX AT 440NM IN 0.1N SODIUM HYDROXIDE	E1% = 28 AT LAMBDA MAX 440NM
RECOMMENDED RETEST SOP QC-12-006	5 YEARS	APRIL 2011
QC RELEASE DATE		APRIL 2006

[redacted]
Analytical Services
St. Louis, Missouri USA

FINAL REPORT AMENDMENT

There is no final report amendment at this time.

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