Title

Characterization of the PAT/*pat* protein batch n°1995 produced in *Escherichia coli*

Study Director

Sofie Moens

Completed On

March 5th, 2012

Test Facility

Bayer CropScience N.V.
Regulatory Science
Protein and Product Characterization
Technologiepark 38
B-9052 Gent
Belgium

Study number

BBS11-010

M-426742-01-1

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The undersigned hereby declare that the work to which this report refers was performed according to the procedures herein described and this report provides an accurate record of the results obtained. All phases of the study were conducted in accordance with the Good Laboratory Practice Standards as specified in the OECD/EU principles of Good Laboratory Practice.

Study Director [redacted] 05/03/2012

Protein and Product Characterization
Regulatory Science

Date

STUDY IDENTIFICATION PAGE

Study Initiation Date:	19/12/2011
Experimental Start Date:	20/12/2011
Experimental Termination Date:	13/01/2012
Study Completion Date:	05/03/2012

Test Facility:

Bayer CropScience N.V.
Regulatory Science
Protein and Product Characterization
GLP Test Facility
Technologiepark 38
9052 Gent – Belgium
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Test Facility Manager:

[redacted]
Address: see Test Facility
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Study Director:

[redacted]
Address: see Test Facility
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Study Personnel:

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Test Site 1: (Peptide mapping)	SGS M-Scan SA 12 chemin des Aulx 1228 Plan-les-Ouates, Switzerland [redacted]
Principal Investigator – Test site 1:	[redacted] Address and Tel. See Test Site 1 [redacted]
Test Site Management – Test site 1:	[redacted] Address and Tel. See Test Site 1 [redacted]
Test Site 2: (N-terminal sequencing)	SGS M-Scan Ltd 3 Millars Business Centre Fishponds Close Wokingham RG41 2TZ, UK [redacted]
Principal Investigator – Test site 2:	[redacted] Address and Tel. See Test Site 2 [redacted]
Test Site Management – Test site 2:	[redacted] Address and Tel. See Test Site 2 [redacted]
Sponsor Representative:	[redacted] Address: see Test Facility Tel: +32 9-243 06 53 Fax: +32 9-224 06 94 [redacted]

QUALITY ASSURANCE STATEMENT

Report **BBS11-010** Date: 05/03/2012
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Quality Assurance (GLP)

Quality Assurance Statement

Title: **Characterization of the PAT/*pat* protein batch n°1995 produced in *Escherichia coli***

Study: BBS11-010

This study was periodically inspected and properly signed records of these inspections were submitted to Test Facility Management and to the Study Director as listed below. This report has been audited by the GLP Quality Assurance Unit. The reported results accurately reflect the original data of the study.

Phase of Study	Inspection (at TF)	Reporting to TF	Reporting to TS
Study plan	19 - 20 DEC 2011	20 DEC 2011	Not applicable
Study conduct - processing	10 JAN 2012	10 JAN 2012	Not applicable
Study plan amendment	24 JAN 2012	24 JAN 2012	26 JAN 2012
Raw data	*02 - 08 FEB 2012*	*10 FEB 2012*	*Not applicable*
Draft final report	13 - 16 FEB 2012	16 FEB 2012	Not applicable
Final report	01 - 02 MAR 2012	02 MAR 2012	Not applicable

Sofie Tanghe

GLP Quality Assurance

(of Test Facility)

[redacted]

05/03/'12

See also Test Site QA Statements in Appendices 3 en 4

APPROVALS PAGE

Study Director [redacted] 05/03/2012
Date

Test Facility Manager [redacted] 5/3/12
Date

Sponsor representative [redacted] 05/03/2012
Date

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SUMMARY

In this study, the identity of the PAT/*pat* protein Batch n° 1995 produced in *Escherichia coli* was confirmed by means of SDS-PAGE, western blotting, glycostaining, peptide mapping and N-terminal sequencing.

The deduced molecular weight of 20.6 kDa was experimentally confirmed for the PAT/*pat* protein batch 1995 by SDS-PAGE. Western blot analysis showed that batch 1995 was immunoreactive when using antibodies raised against the PAT/*pat* protein. Glycostaining showed that this protein batch in not glycosylated. The identity of the protein was confirmed by peptide mapping. A coverage of 76.5 % was obtained. The theoretical N-terminal sequence (MSPER) was confirmed by sequencing by automated Edman degradation. Therefore, we can conclude that the identity of this batch of PAT/*pat* was confirmed.

1. OBJECTIVE

In this study, the characterization of the PAT/*pat* protein batch n° 1995 produced in *Escherichia coli* (*E. coli*) was performed by means of SDS-PAGE, western blotting, glycostaining, peptide mapping and N-terminal sequencing.

2. TEST ITEM, REFERENCE ITEM AND STANDARDS

2.1 Test item

The subject of this study was the PAT/*pat* protein batch 1995 produced in *E. coli.*

Test item ID: T52-01
Expiry Date: Lyophilized powder: November 2012
In solution: used the same day of dissolving
Storage conditions: Dry and ultra frozen (<-30°C) i f lyophilized.
The lyophilized batch can be stored at -20°C up to 2 weeks.
Theoretical Molecular weight: 20618 Da
Molar Extinction coefficient: 44380
Theoretical Amino Acid sequence:
MSPERRPVEIRPATAADMAAVCDIVNHYIETSTVNFRTEP
QTPQEWIDDLERLQDRYPWLVAEVEGVVAGIAYAGPWKAR
NAYDWTVESTVYVSHRHQRLGLGSTLYTHLLKSMEAQGFK
SVVAVIGLPNDPSVRLHEALGYTARGTLRAAGYKHGGWHD
VGFWQRDFELPAPPRPVRPVTQI
Appearance: White powder
Protein (Bradford – test): 0.90 mg/mg of lyophilized substance (Bogner, 1995, Appendix 1)
Purity: > 98 % (Bogner, 1995, Appendix 1)

2.2 Reference item

No reference items were used in this study.

2.3 Standards

The molecular weight marker 'Precision Plus Protein™ Dual Xtra Standards Color Marker' (Bio-Rad, #161-0377)' was used as standard in this study. The stock solution of Precision Plus Protein™ Dual Xtra Standards Color Marker was stored in the freezer. An aliquot of the stock solution was stored refrigerated and was used as a working solution.

The Glycoprotein mix used in the glycostaining was a mixture of the glycosylated alpha-one acidic glycoprotein (44 kDa – 1.6 µg/µL) and the non-glycosylated proteins phosphorylase B (97 kDa – 3.52 µg/µL) and carbonic anhydrase (29 kDa – 1.06 µg/µL). The solution of the Glycoprotein mix was stored in the freezer.

3. EXPERIMENTAL DESIGN

All experiments were performed according to the actual valid SOP. An overview of the SOPs used at the test facility is presented in Appendix 2.

3.1 Dissolving of the test item

One 5.3 mg aliquot of the test item was dissolved in 5.3 mL of protein specific buffer (20 mM Tris pH 7.5; 5 mM EDTA; 1 mM DTT; 100 mM NaCl). The mixture was gently vortexed and shaken during approximately 1 hour in the cold room before use. Once dissolved, the tube was kept on ice or in the refrigerator. As described by Bogner (1995, Appendix 1), 5 mg of powder corresponds to 4.5 mg of protein. After dissolving 5.3 mg in 5.3 mL of buffer the concentration of the dissolved protein was 0.9 mg/mL.

3.2 Molecular weight determination of the test item

To determine the molecular weight, the dissolved test item was analyzed by SDS-PAGE. A NuPAGE® NOVEX Bis-Tris 4-12% (Invitrogen) was used in combination with NuPAGE® 1x MES SDS gel running buffer (Invitrogen).
Loading order of gel G/11-010/01:

Lane 1: 5 µL of the Precision Plus Protein™ Dual Xtra Standards Color Marker
Lane 2: 1 µg of the dissolved PAT/*pat*
Lane 3: 1 µg of the dissolved PAT/*pat*
Lane 4: 5 µL of the Precision Plus Protein™ Dual Xtra Standards Color Marker
Lane 5: 1 µg of the dissolved PAT/*pat*
Lane 6: 1 µg of the dissolved PAT/*pat*
Lane 7: 5 µL of the Precision Plus Protein™ Dual Xtra Standards Color Marker
Lane 8: 1 µg of the dissolved PAT/*pat*
Lane 9: 1 µg of the dissolved PAT/*pat*
Lane 10: 5 µL of the Precision Plus ProteinTM Dual Xtra Standards Color Marker

The electrophoresis was run at a constant voltage of 160 V for approximately 50 minutes.
After electrophoresis, the proteins were stained with SimplyBlue™ Safestain (Invitrogen). The stained gel was digitized using the G:Box BioImaging system from Syngene.

The molecular weight of the PAT/*pat* protein was determined using the GeneTools software (Syngene). In order to obtain a linear regression curve, the molecular weight proteins of 250 kDa, 150 kDa and 2 kDa were not taken into account. The molecular weight of the PAT/*pat* protein was calculated using the obtained linear regression curve and the migration distance of the protein.

The stained gel and 2 sheets of cellophane were soaked in acrylamide gel drying solution. The gel was stretched between both cellophane sheets using the gel drying cassette of the DryEase Mini-Gel Drying system (Invitrogen). The gel was dried overnight.

3.3 Immunoreactivity of the test item by means of western blot

To determine the immunoreactivity of the test item, the dissolved test item was loaded on a NuPAGE® NOVEX Bis-Tris 4-12% (Invitrogen) in combination with NuPAGE® 1x MES SDS gel running buffer (Invitrogen).
Loading order of gel G/11-010/02:

Lane 1: 5 µL of the Precision Plus Protein™ Dual Xtra Standards Color Marker
Lane 2: 0.3 µg of the dissolved PAT/*pat*
Lane 3: 0.1 µg of the dissolved PAT/*pat*
Lane 4: 0.03 µg of the dissolved PAT/*pat*
Lane 5: 5 µL of the Precision Plus Protein™ Dual Xtra Standards Color Marker

The gel was run at a constant voltage of 160 V during 50 minutes. After electrophoresis, the proteins were transferred to a PVDF membrane.
The membrane M/11-010/02 was developed using the AP Conjugate Substrate Kit from Bio-Rad. In this technique two antibodies were used:

- Primary antibody: Rabbit anti-PAT/*pat* (batch n° N.100608). The antibody was used in a 1:2 0 000 dilution.
- Secondary antibody: Goat anti-rabbit-Alkaline Phosphatase (Sigma cat # A8025). The antibody was used at a 1:7000 dilution.

The membrane was digitized using a scanner.

3.4 Analysis for glycosylation

The dissolved test item, positive control and glycoprotein mix were loaded on two identical NuPAGE® NOVEX Bis-Tris 4-12% (Invitrogen) in combination with NuPAGE® MES SDS gel running buffer (Invitrogen).

Loading order of gel G/11-010/03 and G/11-010/04:

Lane 1: 5 µL of the Precision Plus Protein™ Dual Xtra Standards Color Marker
Lane 2: 8 µg Horseradish Peroxidase (positive control)
Lane 3: 4 µg of the dissolved PAT/*pat*
Lane 4: 4 µg of the dissolved PAT/*pat*
Lane 5: 5 µL of Glycoprotein mix

Five µL of the Glycoprotein mix contained equimolar amounts of the proteins, i.e. 8 µg of the glycosylated alpha-one acidic glycoprotein, 17.6 µg of the the non-glycosylated phosphorylase B and 5.3 µg of the non-glycosylated carbonic anhydrase. These amounts correspond to 0.18 nmoles of the respective proteins. An equimolar amount of the Horseradish Peroxidase was loaded.

Four µg of the PAT/*pat* protein corresponds to 0.19 nmoles.

The electrophoresis was run at a constant voltage of 160 V for approximately 50 minutes.

After electrophoresis, the proteins in both gels were stained using the Glycoprotein detection Kit from Sigma. Gel G/11-010/04 was stained omitting the oxidation step to detect non-specific staining. Afterwards both gels were coomassie stained with SimplyBlue™ Safestain (Invitrogen) to confirm the presence of the proteins. The gels were dried afterwards.

The glycostained and coomassie stained gels (G/11-010/03 and G/11-010/04) were digitized using a scanner.

3.5 Mass spec peptide mapping (Test Site 1)

An aliquot of the lyophilized protein was shipped on dry ice to the PI at SGS M-Scan SA (Switzerland) for peptide mapping. An aliquot of the protein specific buffer (20 mM Tris pH 7.5; 5 mM EDTA; 1 mM DTT; 100 mM NaCl) was included in the shipment. The PAT/*pat* protein sample (5.1 mg) was dissolved in 5.1 mL of protein specific buffer. The dissolved sample solution was reduced, alkylated and buffer exchanged. Afterwards this sample was digested with trypsin and subjected to LC-UV-MS peptide mapping as described in the delegated phase report N° 1201/112 00 (Appendix 3). The identity of the protein is confirmed if at least 40 % coverage is obtained in the peptide mapping.

3.6 N-terminal sequencing (Test Site 2)

An aliquot of the lyophilized protein was shipped on dry ice to the PI at SGS M-Scan Ltd (UK) for N-terminal sequencing. An aliquot of the protein specific buffer (20 mM Tris pH 7.5; 5 mM EDTA; 1 mM DTT; 100 mM NaCl) was included in the shipment. The PAT/*pat* protein sample (5.2 mg) was reconstituded using 5.2 mL of protein specific buffer. An aliquot of the sample solution, equivalent to approximately 50 pmoles of protein, was purified, loaded on a PVDF membrane and subjected to N-terminal sequencing by Edman Degradation as described in the delegated phase report N° 1201/22961 (Appendix 4). The identity of the protein was confirmed if the 5 sequenced amino-acids at the N-terminal were correct.

4. RESULTS AND DISCUSSION

4.1 Molecular weight determination

The molecular weight of the PAT/*pat* protein was determined using the photograph with ID number G/11-010/01. A picture of gel G/11-010/01 is shown in Figure 1.

The molecular weight was calculated to be 18.8 ± 2.1 kDa. This fits with the deduced molecular weight of the PAT/*pat* protein of 20.6 kDa.

4.2 Immunoreactivity analysis

A picture of the western blot results is presented in Figure 2. The western blot analysis revealed in each sample the expected PAT/*pat* band, confirming the immunoreactivity of the test item. An additional fragment of approximately 40 kDa was observed. This fragment is probably a dimer. A weak fragment of approximately 15 kDa was also observed. It is highly likely that this fragment is a degradation product of the PAT/*pat* protein.

4.3 Glycosylation analysis

A picture of the glycostained gel G/11-010/03 is shown in Figure 3, Panel A. A picture of the coomassie stained gel is presented in Figure 3, Panel B.

After glycostaining, the Horseradish Peroxidase (positive control) and the glycosylated protein alpha-one acidic glycoprotein of the Glycoprotein mix were visualized as bright bands on the gel (Figure 3A lanes 2 and 5). A weak background signal is detected for the non-glycosylated proteins of the Glycoprotein mix, phosphorylase B and carbonic anhydrase (Figure 3A lane 5).

The coomassie stain of the same gel (Figure 3B) showed the presence of the PAT/*pat* protein in lanes 3 and 4. All fragments of the glycoprotein mix (lane 5) and the positive control (lane 2) were also detected.

Since ample protein was loaded on gel and since the intensity of the signal obtained for the PAT/*pat* protein (Figure 3B lanes 3 and 4) is less than the obtained signal for the non-glycosylated proteins of the glycoprotein mix, it is confirmed that this PAT/*pat* protein batch is not glycosylated.

Gel G/11-010/04 was used to check for non-specific staining. After the glycostaining omitting the oxidation step, non-specific staining was not detected (data not shown).

4.4 Peptide mapping

Peptides of a trypsin digest of the PAT/*pat* protein batch 1995 were analyzed using LC-UV-MS. The analysis and results are described in the delegated phase report 1201/11200 (Appendix 3). Table 1 provides a summary of the mapped peptides and their theoretical and obtained masses.
The percentage coverage determined for the PAT/*pat* protein batch 1995 was 76.5%. These data confirm the identity of this protein batch.

Table 1: Summary of the peptide mapping

Peptide Residue numbers	Theoretical mass (protonation state)	Obtained masses for PAT/*pat* batch 1995
1-5	619.3 (+1)	619.3
6-11	769.5 (+1)	769.5
12-37	2809.3 (+1)	ND
38-52	928.9 (+2)	928.9
53-56	531.3 (+1)	531.3
57-73	1836.0 (+1)	ND
74-78	558.3 (+1)	558.3
79-80	246.2 (+1)	246.2
81-96	964.0 (+2)	964.0
97-99	440.2 (+1)	440.3
100-112	708.4 (+2)	708.4
113-120	449.2 (+2)	449.2
121-135	761.9 (+2)	761.9
136-145	565.8 (+2)	565.8
146-149	446.3 (+1)	446.3
150-154	509.3 (+1)	509.3
155-166	741.3 (+2)	741.4
167-183	966.5 (+2)	966.6

ND: not detected

4.5 N-terminal sequencing

The N-terminal sequence determination of the PAT/*pat* protein batch 1995 is described in the delegated phase report 1201/22961 (Appendix 4). Five residues were sequenced by Automatic Edman degradation. The obtained N-terminal sequence (MSPER) is consistent with the theoretical amino acid sequence.

5. CONCLUSION

In this study, the identity of the PAT/*pat* protein Batch n° 1995 produced in *Escherichia coli* was confirmed by means of SDS-PAGE, western blotting, glycostaining, peptide mapping and N-terminal sequencing.

The deduced molecular weight of 20.6 kDa was experimentally confirmed for the PAT/pat protein batch 1995 by SDS-PAGE. Western blot analysis showed that batch 1995 was immunoreactive when using antibodies raised against the PAT/*pat* protein. Glycostaining showed that this protein batch is not glycosylated. The identity of the protein was confirmed by peptide mapping; 76.5 % coverage was obtained. The theoretical N-terminal sequence (MSPER) was confirmed by sequencing by Automated Edman Degradation. Therefore we can conclude that the identity of this batch of PAT/*pat* is confirmed.

6. ARCHIVING

The study plan, the amendments and deviations, the study data and the original final report will be archived in study file BBS11-010 in the Bayer CropScience N.V. GLP Test Facility document archive.

An aliquot of 5.5 mg of test item T52-01 was archived in the test and reference item archive ultrafreezer of the GLP Test Facility Archive.

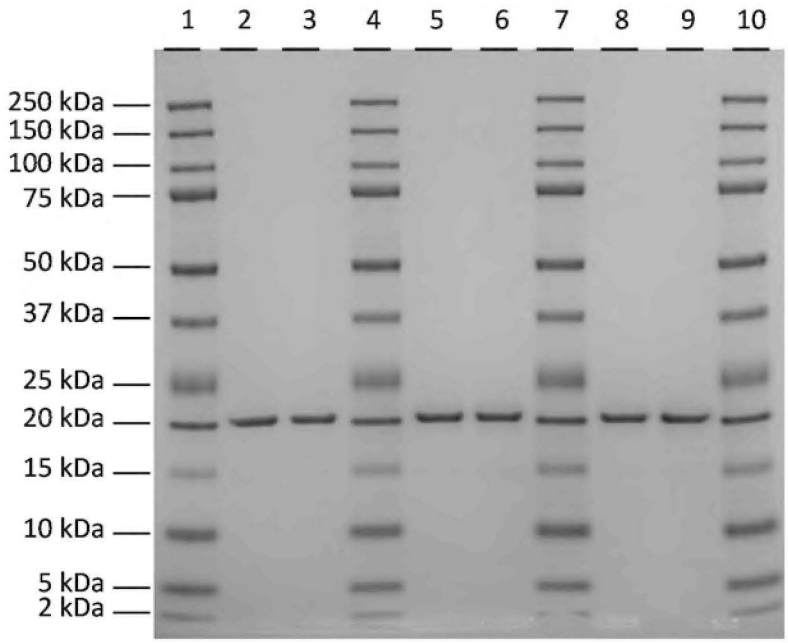


Figure 1: Determination of the molecular weight of test item (SDS-PAGE)

Gel ID G/11-010/01

Loading order : Lane 1: 5 µL of the Precision Plus Protein™ Dual Xtra Standards Color Marker
Lane 2: 1 µg of the dissolved PAT/*pat*
Lane 3: 1 µg of the dissolved PAT/*pat*
Lane 4: 5 µL of the Precision Plus Protein™ Dual Xtra Standards Color Marker
Lane 5: 1 µg of the dissolved PAT/*pat*
Lane 6: 1 µg of the dissolved PAT/*pat*
Lane 7: 5 µL of the Precision Plus Protein™ Dual Xtra Standards Color Marker
Lane 8: 1 µg of the dissolved PAT/*pat*
Lane 9: 1 µg of the dissolved PAT/*pat*
Lane 10:5 µL of the Precision Plus Protein™ Dual Xtra Standards Color Marker

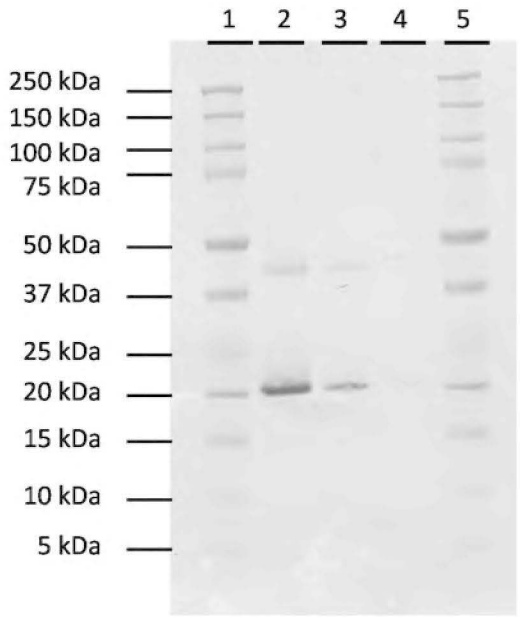
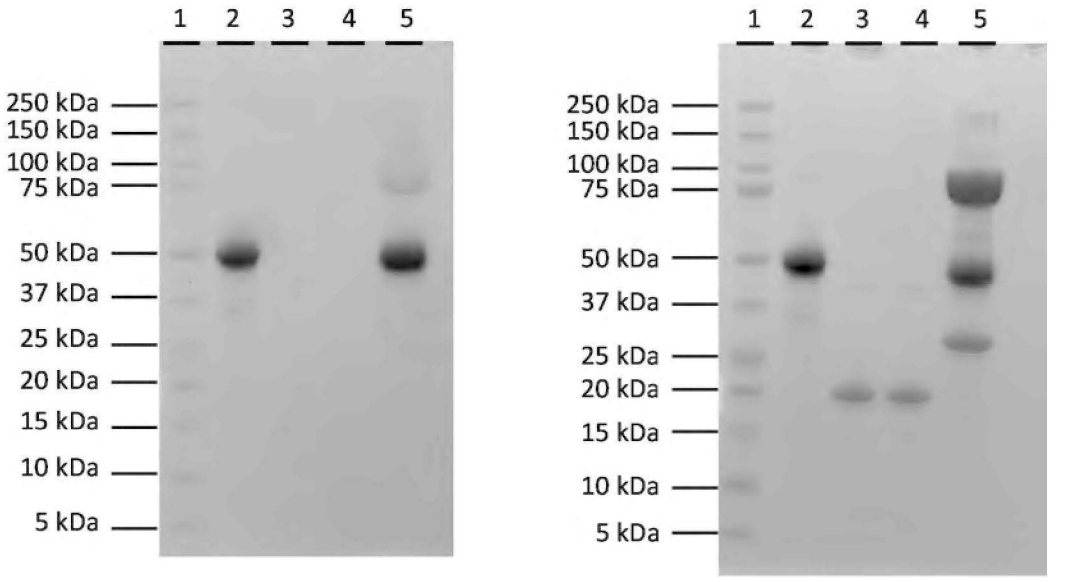


Figure 2: Western blot of the test item

Membrane ID M/11-010/02

Loading order: Lane 1: 5 µL of the Precision Plus Protein™ Dual Xtra Standards Color Marker
Lane 2: 0.3 µg of the dissolved PAT/*pat*
Lane 3: 0.1 µg of the dissolved PAT/*pat*
Lane 4: 0.03 µg of the dissolved PAT/*pat*
Lane 5: 5 µL of the Precision Plus Protein™ Dual Xtra Standards Color Marker



PANEL A: glycostained gel PANEL B: coomassie stained gel

Figure 3: Glycosylation analysis of the test item

Gel ID G/11-010/03

Loading order: Lane 1: 5 µL of the Precision Plus Protein™ Dual Xtra Standards Color Marker
Lane 2: 8 µg Horseradish Peroxidase (positive control)
Lane 3: 4 µg of the dissolved PAT/*pat*
Lane 4: 4 µg of the dissolved PAT/*pat*
Lane 5: 5 µL of Glycoprotein mix

Appendix 1: Certificate of Analysis (Bogner, 1995)

Boehringer Mannheim GmbH, Penzberg
Biotechnology Operations Quality Control

19.09.1995

Certificate of Analysis

Cat.-no

Product sample for AGREVO

Order no.

Quantity 220 g Lyophilized powder corresp. to 198 g protein

lot sample AG 02

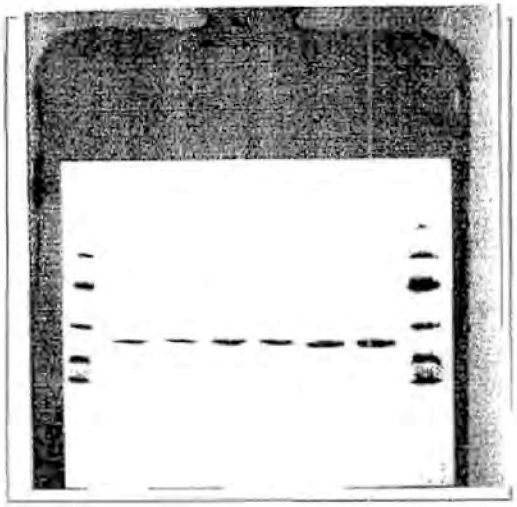
Parameter	Results
Apearance	white lyophilized substance
Contents	K-Phosphate, Protein
Activity	4.6 U/mg lyophilized substance
Specific Activity	5.1 U/mg protein
Protein (Bradford-test)	0.90 mg/mg lyophilized substance
Phosphate (calc. as $K_2HPO_4 \times 3\ H_2O$)	1.8 %
Purity (SDS-PAGE)	> 98 %
Bioburden	1140 CFU/g Lyo.
Endotoxines (Limulus) (corresp. to < 0.75 µg Lipopolisaccharides /g Lyo.)	< 6000 EU/g

Boehringer Mannheim GmbH, Penzberg
Biotechnology Operations Quality Control

sign.: [redacted]

Boehringer Mannheim GmbH, Penzberg **19.09.1995**
Biotechnology Operations Quality Control

SDS-Page



Marker 0.09 µg/slot 0.15 µg/slot 0.23 µg/slot Marker

Sample AG 02: SDS-PAGE, reducing conditions

Appendix 2: Overview of analytical SOPs used at the test facility.

SOP	Title	Reference
BBS 07/42/02	Fragment analysis using the genetools software	Genetools user manual - Syngene
BBS 07/63/05	Sodium Dodecyl Sulphate Poly-Acrylamide Gel Electrophoresis (SDS-PAGE)	- Precise™ Protein Gels Instruction manual (1472.5) – Thermo Scientific. - NuPAGE Technical guide (AM-1001 Version E) - Invitrogen. - Criterion™ Cell – Instruction Manual – Catalog Number 165-6001 – BIO-RAD.
BBS 07/64/04	Electrotransfer of proteins to membranes	- Instruction manual Mini Trans-Blot® Electrophoretic Transfer Cell (Version M1703930 Rev.E) – BioRad
BBS 07/65/03	Western blotting	
BBS 07/81/00	Coomassie staining	- Instructions Gelcode® Blue Stain Reagent, Pierce, version 0714.3 – catalogue #24590 and 24592 - User manual SimplyBlue™ Safestain for fast, sensitive, and safe Coomassie® G-250 staining of proteins from Invitrogen. Catalog nos LC6060, LC6065 version MAN0000735. - User manual DryEase® Mini-Gel Drying System for even, crack-free drying of mini-gels from Invitrogen. Catalog nos. NI2387, NI2380 version MAN0000730.
BBS 07/92/00	Procedure for the GlycoPro glycoprotein detection kit	Glycoprotein Detection Kit GLYCOPRO – Technical bulletin of Sigma.

Appendix 3: Phase report of the peptide mapping

SGS M-Scan

Analytical Phase Report

Study title :	**Characterization of the PAT/*pat* protein batch n° 1995 produced in *Escherichia coli***
Test item :	**PAT/*pat* protein, T52-01**
Batch No. :	**1995**
Analytical Phase Title :	**Mass Spectrometry characterization by peptide mapping**
Study No. :	**BBS11-010**
Analytical Phase Plan No. :	**G1111110**
Analytical Phase Report No. :	**1201/11200**
Document status :	**Final**
Date :	**07 FEB 2012**

Report prepared and issued by : [redacted] Date 07 FEB 2012

Report reviewed by : [redacted] Date 07 FEB 2012

CONFIDENTIAL

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M-SCAN IS NOW PART OF SGS, THE WORLD'S LEADING INSPECTION, VERIFICATION, TESTING AND CERTIFICATION COMPANY.

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Member of the SGS Group (SGS SA)

GENERAL INFORMATION

Test Facility :

Bayer CropScience N.V.
Regulatory Science –
Protein and Product Charcterization
GLP Test Facility
Technologiepark 38
B-9052 Ghent

Study Director :

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Test Facility Quality Assurance:

[redacted]

Test Site :

SGS M-Scan SA
12 chemin des Aulx
CH-1228 Plan-les-Ouates

Principal Investigator :

[redacted]

Test Site Quality Assurance :

[redacted]

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Study No.: BBS11-010
Analytical Phase Plan No. G1111110
Analytical Phase Report No. 1201/11200

SGS M-Scan SA

PRINCIPAL INVESTIGATOR STATEMENT

Study No. :	**BBS11-010**
Analytical Phase No. :	**G1111110**
Test item :	**PAT/*pat* protein, T52-01**
Batch No. :	**1995**
Phase Report No. :	**1201/11200**
Principal Investigator :	[redacted]
Study Title :	**Characterization of the PAT/*pat* protein batch n° 1995 produced in *Escherichia coli***
Analytical Phase Title :	**Mass Spectrometry characterization by peptide mapping**

I, the undersigned, acknowledge that the work described in this phase report was conducted in compliance with the requirements of the Swiss GLP ordinance (reference : Document OBPL No. 813.112.1, May 18th, 2005) which is based on the OECD Principles of Good Laboratory Practice, as revised in 1997, and adopted November 26th, 1997 by decision of the OECD Council [C(97) 186/Final], and in compliance with multi-site rules as described in "OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring (The Application of the OECD Principles of GLP to Organisation and Management of Multi-Site Studies, Number 13).

The phase report describes the procedures used and is an accurate reflection of the raw data generated during the performance of the analytical phase.

Signed [redacted] Principal Investigator

Date 07 FEB 2012

QUALITY ASSURANCE STATEMENT

Study No. : **BBS11-010**

Analytical Phase No. : **G1111110**

Test item : **PAT/*pat* protein, T52-01**

Batch No. : **1995**

Phase Report No. : **1201/11200**

Principal Investigator : [redacted]

Study Title : **Characterization of the PAT/*pat* protein batch n° 1995 produced in *Escherichia coli***

Analytical Phase Title : **Mass Spectrometry characterization by peptide mapping**

Various aspects of this analytical study phase were subjected to an audit by the Quality Assurance Unit of the test site. The test site is an accredited GLP compliant laboratory.
The audit dates are given below :

Dates of QA Inspections	Phase inspected	Dates of reporting to Test Site Management and Principal Investigator	Dates of reporting to Study Director
16 DEC 2011	Phase Plan	16 DEC 2011	16 DEC 2011
21 DEC 2011	Sample receipt and registration	22 DEC 2011	22 DEC 2011
22 DEC 2011	Sample preparation and analysis conditions	05 JAN 2012	09 JAN 2012
30 JAN 2012	Study Plan Amendment BBS11-010-A1	30 JAN 2012	31 JAN 2012
03/06 FEB 2012	Draft Phase Report	06 FEB 2012	07 FEB 2012
07 FEB 2012	Final Phase Report	07 FEB 2012	07 FEB 2012

As far as can be reasonably established the phase report was considered to be an accurate reflection of the raw data.

Signed [redacted]
Quality Assurance

Date 07 FEB 2012

Study No.: BBS11-010
Analytical Phase Plan No. G1111110
Analytical Phase Report No. 1201/11200

SGS M-Scan SA

LIST OF ABBREVIATIONS

DTT =	DL-Dithiothreitol
ES =	Electrospray
LC =	Liquid Chromatography
MS =	Mass Spectrometry
SOP =	Standard Operating Procedures
UV =	Ultra Violet

LIST OF SOPs

B-002/4	Enzymic Digestion using Trypsin.
B-009/3	Reduction.
B-010/3	Alkylation using vinylpyridine.
B-014/2	Peptide Mapping Interpretation.
B-015/2	Buffer exchange/concentration of sample in solution with Centrifugal filter.
BI-028/1	Procedure to use the Digital Dry Bath (model FB15103) (Fisher Scientific).
GP-001/16	Receipt of samples.
GP-009/8	Procedure for Experimental Data Recording: Working Files.
GP-010/6	Preparation, Issue and Review of Reports.
GP-013/8	Waste Disposal / Disposal of samples.
GP-014/10	Archives.
GP-021/5	Deviation procedure.
MS-012/6	Procedure for preparing solvent A.
MS-013/7	Procedure for preparing solvent B.
MS-402/7	General Operation of the Micromass Q-Tof *micro*™ Quadrupole Time of Flight Mass Spectrometers (YA101 and YB355).
MS-404/8	Generation of a calibration on the Micromass Q-Tof *micro*™ spectrometers.
MS-410/4	Procedure for injecting samples on LC systems coupled to the Q-Tof *micro*™ mass spectrometers.

PHASE PLAN ADHERENCE

The SOP BI-028/1 (Title: "Procedure to use the Digital Dry Bath (model FB15103) (Fisher Scientific)") used for the sample preparation was not described in the "Sample preparation" section of the Analytical Phase Plan.
It was added in the section "Sample preparation" as documented by the Study Director in the amendment No. BBS11-010-A1 to the Study Plan BBS11-010.

ANALYTICAL PHASE TIME-SCALE

Work commencement date : 22 DEC 2011
Work completed date : 23 DEC 2011
Draft report : 24 JAN 2012
Final report : signature date.

KEY PERSONNEL

[redacted]

DISTRIBUTION LIST

Study Director (copy)
Test Site Study File (original)

DATA COLLECTION AND RECORDING OF OBSERVATIONS

A copy of the Study Plan, the original Analytical Phase Plan, all descriptions of experimental details, methods, equipment and raw materials used together with all printouts and chromatograms, defined as data, were collected in a working file labelled with the appropriate study and job numbers as described in SOP GP009 at the Test Site.

ARCHIVING, DELIVERY AND RETURN OF DATA

On completion of the analytical phase, certified copies of all data collected in the working file will be transferred to the Study Director.
The working file and the original final Analytical Phase Report will be stored in an archive at SGS M-Scan SA Test Site as described in SGS M-Scan SOP GP014 for at least 10 years.
At the end of this 10 year period the Study Director will be contacted with regards to future archiving arrangements.

SAMPLE DISPOSAL

One month after completion of the analytical phase, the Study Director will be contacted with regards to remaining sample material disposal (SOP GP013).
Within three months as from Study Director approval for the disposal, SGS M-Scan SA will organize the destruction of the remaining sample material and will confirm such destruction to the Study Director by e-mail.

1. OBJECTIVE

The aim of the analytical phase was to examine by LC-UV-MS an enzymatic digest of a protein sample in order to establish its peptide map.

2. SAMPLES

The samples described below were submitted to SGS M-Scan SA and registered according to SOP GP001.

Test Item

Identification :	PAT/*pal* protein
Batch No. :	1995
ID :	T52-01
Supplier :	Bayer CropScience N.V.
Form :	Lyophilized
Sample amount :	5.1mg
SGS M-Scan No. :	57630
Expiry / Retest date :	November 2012
Storage conditions :	Dry and ultra frozen (≤ -70°C) if lyophilized 2-8°C after dissolving or thawing (expired after 1 day)

The following expected primary amino acid sequence was supplied by the Study Director and converted to a format compatible with BioLynx™ :

Average Mass = 20618.3573, Monoisotopic Mass = 20605.4684
N-Terminus = H, C-Terminus = OH

```
1    MSPER RPVEI RPATA ADMAA VCDIV NHYIE TSTVN FRTEP QTPQE WIDDL
51   ERLQD RYPWL VAEVE GVVAG IAYAG PWKAR NAYDW TVEST VYVSH RHQRL
101  GLGST LYTHL LKSME AQGFK SVVAV IGLPN DPSVR LHEAL GYTAR GTLRA
151  AGYKH GGWHD VGFWQ RDFEL PAPPR PVRPV TQI
```

Protein specific buffer

Identification :	20mM Tris pH 7.5, 5mM EDTA, 1mM DTT, 100mM NaCl
Supplier :	Bayer CropScience N.V.
Batch No. :	Not specified
Sample amount :	10mL
SGS M-Scan No :	57631
Expiry / Retest date :	09 January 2012
Storage conditions :	Room temperature

The supplier was responsible for the purity and the stability of the samples.

3. MATERIALS

3.1 Equipment

Analyses were performed using the qualified equipment described on next page.

Instrument	Model	Manufacturer
Mass spectrometer	Q-Tof *micro*™ (YB355)	Waters
HPLC Integrated System	HP1100	Agilent

3.2 Consumables

All consumables used satisfied the requirements for handling both chemical and biological materials.

3.3 Chemicals

All solvents and reagents used were of analytical grade or better.

3.4 LC column

Supplier	Stationary phase	Cat No.	Dimension IDxL(mm)
Grace / Vydac	218TPC$_{18}$ 5µ	218TP5215	2.1 x 150

4 METHODS

The methods and procedures which were used are described in the current versions of SGS M-Scan SA Standard Operating Procedures.

4.1 Sample preparation

The sample as received was dissolved in 5.1mL of 20mM Tris pH 7.5, 5mM EDTA, 1mM DTT, 100mM NaCl buffer.

4.1.1 Reduction / alkylation

An aliquot of the sample solution was reduced with DL-Dithiothreitol (SOPs B009 and BI028) and alkylated with 4-Vinylpyridine (SOP B010).

4.1.2 Buffer exchange / removal of reagents excesses

An aliquot of the reduced and alkylated sample solution was buffer exchanged against 0.5% Formic acid in 50mM Ammonium Bicarbonate using Amicon Ultra-0.5mL Ultracel®-3K membranes, centrifugal filters devices (SOP B015). The pH of the resulting solution was then adjusted to ~ 8.0 by adding an appropriate volume of 50mM and 1M Ammonium Bicarbonate.

4.1.3 Trypsin digestion

An aliquot of the reduced, alkylated and buffer exchanged sample solution was digested with Trypsin overnight at 37°C (SOPs B002 and BI028).

4.2 Sample analysis

LC-UV-MS analyses were performed on a qualified system consisting of a liquid chromatography Agilent HP1100 integrated system coupled to a Waters Q-Tof *micro*™ (YB355) mass spectrometer (SOP MS402).

4.2.1 Instrument calibration

Calibration of the mass spectrometer was performed using CsI. Mass accuracy was better than ± 0.2Da/e for the major signals observed prior to sample analysis (SOP MS404).

4.2.2 LC-UV-MS peptide mapping

An aliquot of the prepared sample solution was injected on a C_{18} reversed phase column connected to the source of the mass spectrometer (SOP MS410).
A blank analysis (injection of water) was performed prior to the analysis of the prepared sample solution using the same column and the same LC-UV-MS conditions.

Liquid Chromatography conditions

Solvent A : 1mL TFA in 1000mL H_2O (SOP MS012)
Solvent B : 1mL TFA in 10mL H_2O and 990mL CH_3CN (SOP MS013)
Column : Vydac C_{18} 150mm x 2.1mm
Flow rate : set at 300µL/min
Column temperature : set at 40°C
UV detection : set at 215nm
Gradient :

Time (min)	% solvent B
0	0
5	0
55	50
60	90

Mass spectrometry conditions

Acquisition was performed in positive electrospray scanning mode over the m/z range 110-3000.

4.3 Data acquisition and processing

LC-UV-MS data were acquired and processed using MassLynx™ software version 4.1.
Interpretation of the raw data was aided by the use of the BioLynx™ software supplied with the current version of MassLynx™ in conjunction with the sequence supplied by the Study Director and following SOP B014.

5 RESULTS

Date of analysis : 23 DEC 2011
Operator : Audrey Bednarczyk

Results of the detailed processing of the LC-UV-MS analysis of the prepared sample solution are given in table 1.

Table 1 : Summary results of the peptide mapping of the reduced, alkylated and Trypsin digested PAT/*pat* protein sample (ID: T52-01, Batch No. 1995).

UV rt (min)	Mass[1] observed (Protonation state)[2]	Possible assignment	Notes
Approx. 1.5	246.2 (+1) 278.2 (+1) 391.3 (+1) 440.3 (+1)	79-80 n.a. n.a. 97-99	
4.13	No m/z signals which could be peptide related were observed		
5.87	No m/z signals which could be peptide related were observed		
7.11	No m/z signals which could be peptide related were observed		
9.02	509.3 (+1)	150-154	
Approx. 11.4	531.3 (+1)	53-56	
Leading 13.89	No m/z signals which could be peptide related were observed		
13.89	446.3 (+1)	146-149	

Table 1 : (Continued) Summary results of the peptide mapping of the reduced, alkylated and Trypsin digested PAT/*pat* protein sample (ID: T52-01, Batch No. 1995).

UV rt (min)	Mass[1] observed (Protonation state)[2]	Possible assignment	Notes
15.33	619.3 (+1)	1-5	N-terminal
16.08	265.2 (+1) 281.2 (+1) 365.2 (+1)	n.a. n.a. $DTT(VP)_2$	Not peptide related
16.32	No m/z signals which could be peptide related were observed		
17.00	No m/z signals which could be peptide related were observed		
17.82	373.3 (+1) 689.4 (+1)	n.a. n.a.	
18.70	402.3 (+1) 515.3 (+1) 667.3 (+1)	n.a. AT n.a.	
20.32	558.3 (+1)	74-78	
21.33	769.5 (+1)	6-11	
21.58	436.3 (+1)	n.a.	
21.88	449.2 (+2)	113-120	
Approx. 22.2	557.3 (+1)	179-183	C-terminal
23.04	523.3 (+2) 802.5 (+1)	AT n.a.	
24.70	565.8 (+2)	136-145	
25.53	802.4 (+1)	136-142	
Leading 26.44	455.3 (+2)	176-183	
26.44	421.8 (+2) 571.4 (+1) 672.4 (+1)	AT n.a. n.a.	
26.75	428.8 (+2)	AT	
27.07	435.8 (+2)	AT	
27.47	No m/z signals which could be peptide related were observed		
30.58	505.3 (+1) 521.3 (+1) 537.3 (+1)	n.a. n.a. n.a.	Not peptide related
31.98	741.4 (+2) 964.0 (+2)	155-166 81-96	
32.64	898.4 (+2)	n.a.	
33.08	971.0 (+2)	AT	
Leading 33.36	1106.1 (+2)	AT	
33.36	761.9 (+2)	121-135	
35.21	966.6 (+2)	167-183	
35.94	928.9 (+2)	38-52	
Approx. 37.1	708.4 (+2) 1142.1 (+2)	100-112 AT	
47.89	No m/z signals which could be peptide related were observed		

Notes :

The rows in bold correspond to the major UV peaks observed.
Masses were not reported if :

- They were assigned to peptide fragment ions generated during the analysis.
- They were assigned to signals produced from various identified source of the system (chemical background, buffer, ...).
- They were already reported in the previous or the next series of combined scans.
- They are lower than our reporting threshold.

1) : Masses reported as monoisotopic unless otherwise specified.
2) : If protonation state is reported as +1 or +2, the mass reported corresponds to an m/z ion.
n.a. : Not assigned.
rt : Retention time.
AT : Peptide generated from Trypsin autolysis.

6 CONCLUSION

The LC-UV-MS analysis of the reduced, alkylated and Trypsin digest of the PAT/*pat* protein sample (ID: T52-01, Batch No. 1995) produced distinct m/z signals which could be assigned to possible peptide fragments released from PAT/*pat* protein sequence supplied by the Study Director.
This allowed 76.5% of the protein sequence to be mapped which is well in accordance with the acceptance criteria defined in the Analytical Phase Plan : "The peptide mapping results will be sufficient if, based on the sequence supplied by the Study Director, 40% sequence coverage is obtained".

The two following peptides were not mapped:
Residues 12-37
Residues 57-73

A series of m/z signals stayed unassigned and would require further work such as MS/MS to be characterized.

Finally, it should be noted that the low intensities of the LC-UV-MS signals generated during the analysis of the sample suggested that part of the intact protein sample could have been lost during one of the sample preparation steps.

Appendix 4: Phase report of the N-terminal sequencing

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STUDY TITLE:	Characterization for the PAT/*pat* protein batch n° 1995 produced in *Escherichia coli*
STUDY PLAN NO:	BBS11-010
ANALYTICAL PHASE TITLE:	N-Terminal Amino Acid Sequencing
TEST SITE:	SGS M-SCAN LIMITED 2-3, Millars Business Centre, Fishponds Close, Wokingham, Berkshire, RG41 2TZ, UK
ANALYTICAL PHASE STUDY PLAN NO:	11121602
STUDY SPONSOR AND TEST FACILITY:	BAYER CROPSCIENCE N.V. Technologiepark 38, 9052 Zwijnaarde (Gent), Belgium
STUDY DIRECTOR:	[redacted]
REPORT NO:	1201/22961

Report prepared by: [redacted] date: 1st Feb 2012

Biochemist (Principal Investigator)

Report reviewed by: [redacted] date: 01st Feb 2012

Biochemist (Test Site Operator)

And by: [redacted] date: 1st Feb 2012

Managing Director (Test Site Management)

M-SCAN IS NOW PART OF SGS, THE WORLD'S LEADING INSPECTION, VERIFICATION, TESTING AND CERTIFICATION COMPANY.

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Member of the SGS Group (SGS SA)

Registered in England No: 1414626 Registered Office: Inward Way Rossmore Business Park Ellesmere Port Cheshire CH65 3EN

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QUALITY ASSURANCE STATEMENT

Report Number: 1201/22961

Analytical Phase Study Number: 11121602

Study Number: BBS11-010

Title: Characterization for the PAT/pat protein batch N° 1995 produced in Escherichia coli.

Quality Assurance reviewed the study in accordance with the Good Laboratory Practice Regulations, 1999 SI 3106 and subsequent amendment (2004) SI No. 994, as administered by the UK Medicine and Healthcare Products Regulatory Agency.

The following phases of the study were inspected and the findings reported to study management:

Date Inspected	Phase	Date written report made to Management and and Principal Investigator	Date written report made to Study Director
16 Dec 2011	Draft Analytical Phase Study Plan	16 Dec 2011	16 Dec 2011
22 Dec 2011	Sample receipt	22 Dec 2011	22 Dec 2011
23 Dec 2011	Sample preparation and N-terminal sequencing	23 Dec 2011	23 Dec 2011
31 Jan 2012	Draft Report	31 Jan 2012	02 Feb 2012

Details of the analytical phase inspected are listed above. Additional laboratory procedures are inspected on a routine basis in accordance with SGS M-Scan Limited standard operating procedures.

Facility inspections are conducted according to an approved schedule as described in SGS M-Scan Ltd standard operating procedures.

As far as can be reasonably established, the report was considered to be an accurate reflection of the raw data generated during the conduct of the study.

Signed [redacted]

(Quality Assurance Unit)

Date: 02 Feb 2012

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PRINCIPAL INVESTIGATOR

STATEMENT OF COMPLIANCE

Report Number: 1201/22961

Title: Characterization for the PAT/*pat* protein batch n° 1995 produced in *Escherichia coli*

Analytical Phase
Study Plan Number: 11121602

The work described in this report was conducted in accordance with the principles laid down in the UK Good Laboratory Practice Regulations, SI 3106 (1999) as amended by SI 994(2004), which are themselves based on the principles of good laboratory practice contained in Annex 2 to the Decision of the Council of the Organisation for Economic Co-operation and Development (OECD), ENV/MC/CHEM (98)17. They are in conformity with, and implement the requirements of, Directives 2004/10/EC and 2004/9/EC.

No deviations from these principles were made and the report describes the procedures used and is an accurate reflection of the raw data generated during the performance of this study.

Signed [redacted]
(Principal Investigator)

Date 1st Feb 2012

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1. INTRODUCTION

Sequencing of the amino-terminal amino acids for a biotechnological/biological product is usually performed using manual or automated gas phase sequencing using Edman chemistry.

2. OBJECTIVE

The aim of this analytical phase study plan was to analyse a supplied sample using automated Edman degradation chemistry and report the N-terminal sequence obtained. The sample was provided by the Study Director as a dry powder. The sample was reconstituted using the buffer supplied by the Study Director, the buffer components were then removed and the sample transferred to a PVDF disc. Edman degradation was performed using an automated pulsed-liquid sequencer. Automated pulsed-liquid sequencing for five residues of the N-terminal sequence was undertaken.

3. EXPERIMENTAL

3.1 Samples

Two vials were received from Bayer on the 22nd December 2011 and were given the unique SGS M-Scan numbers described below:

Sample	SGS M-Scan No.
T52-01 PAT/pat Batch n°1995 BBS11-010 Lyophylised protein 5,2mg 1c UF Exp. date nov.2012(1)	98681
RT, prepared: 21/12/11 Protein Buffer-A Batch Protein Buffer-A-01 Exp. Date: 04/01/12(1) AVR; study: BBS11-010	98682

(1) Expiry date of the samples stored at -70°C. The expiry date of the samples stored at SGS M-Scan facilities was the 5th January 2012, according to Analytical Phase Study Plan number 11121602.

The sample (SGS M-Scan no. 98681) was stored at -20 ± 10°C and buffer vial (SGS M-Scan no. 98682) was stored at room temperature until required. The analysis was performed between the 23rd December 2011 and 5th January 2012.

The Study Director has informed SGS M-Scan Ltd that the sample vial contained 5.2mg of lyophilised powder.

3.2 Sample preparation

The sample (SGS M-Scan number 98681) was thawed at RT. The sample was reconstituted using 5.2ml of buffer (SGS M-Scan no. 98682) to produce a nominal 1mg/ml solution which contained 0.9mg/ml of protein. Ten µl of the sample solution was further diluted to produce a 0.1mg/ml solution with 0.1% *aq.* TFA (v/v). An aliquot equivalent to approximately 50pmoles of the diluted solution was loaded onto a ProSorb® cartridge containing 100µl of 0.1% *aq.* TFA (v/v), and washed three times with 0.1% *aq.* TFA (v/v). The purified sample aliquot was dried onto the PVDF disc from the ProSorb® cartridge, and the disc was removed from the cartridge.

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3.3 N-Terminal sequencing

Pulsed-Liquid N-terminal Sequencing was carried out using an Applied Biosystems Procise 492 automated N-terminal sequencer equipped with HPLC consisting of 140C pumps, a 200 Perkin Elmer series detector, a reverse phase column PTH-C18 cartridge 2.1 x 220 mm.

The PVDF disc containing the sample was loaded onto the Procise Sequencer and five residues were sequenced by Edman degradation. The released phenylthiohydantoin (PTH-) amino-acid derivatives were identified by reversed-phase HPLC analysis.

3.4 Key personnel

E P Seward (Principal Investigator)
R Kara (Test Site Operator)
C Shrimpton (Sample Receipt Operator)
Dr A J Reason (Test Site Management)

3.5 Reagents

All the reagents used during the course of the study are listed in the workfile.

3.6 Archive

A copy of the Study Plan, raw data, working file and one copy of the Final Report will be transferred to the GxP archive at SGS M-Scan Ltd and stored there according to SOP GP013v12. The originals will be transferred to the Study Director.

The sample will be destroyed 1 month after completion of the delegated phase (final phase report sent to the Study Director) according to SOP GP012v6, and the Study Director will be informed by email.

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3.7 Procedures

The following procedures were required for this study:

SOP number	Title
GP015v9	Receipt of samples
PS-001v4	Applied Biosystems Procise N-Terminal Sequencer
PS-002v3	General Operation of the Applied Biosystems Procise N-Terminal Sequencer
PS-004v4	Preparation of PVDF Membrane Samples for Analysis on the Applied Biosystems Procise N-Terminal Sequencer
PS-005v5	Changing Reagent/Solvent Bottles on the Applied Biosystems Procise N-Terminal Sequencer
PS-009v3	Sample Preparation using the Prosorb® Cartridge for Analysis on the Applied Biosystems Procise N-Terminal Sequencer
PS-010v2	Data Processing using the SequencePro software
GP030v1	Laboratory Data Computer Backup
GP013v12	Archives
GP012v6	Waste Disposal

4. RESULTS AND DISCUSSION

4.1 N-Terminal Sequencing of PAT/*pat* protein batch n° 1995 (SGS M-Scan no. 98681)

Pulsed-Liquid sequencing was performed on PAT/*pat* protein batch n° 1995 (SGS M-Scan no. 98681). Five residues were sequenced by Automatic Edman degradation. The data obtained from the analysis is summarised in Table 1.

Table 1: N-Terminal Sequencing of PAT/*pat* protein batch n° 1995 (SGS M-Scan no. 98681)

Residue Number	SGS M-Scan no. 98681 PTH-AA Sequence Observed
1	M
2	S
3	P
4	E
5	R

The data confirms the presence of one N-terminal peptide sequence, consistent with the sequence provided by the Study Director.

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5. CONCLUSION

Analysis of the N-terminal sequence for the sample

Five residues were sequenced from the N-terminus of the supplied sample by Edman degradation performed on a Procise Automatic Protein Sequencer 492.

The data obtained for the sample confirms the following N-terminal sequence:

Sample	SGS M-Scan No.	Sequence
Sequence supplied by Study Director		MSPER
PAT/*pat* protein batch n° 1995	98681	MSPER

The N-terminus of the sample was consistent with the sequence supplied by the Study Director.