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(54) Title: A METHOD FOR CONTROLLING PROTEOLYTIC DEGRADATION OF RECOMBINANT PROTEINS

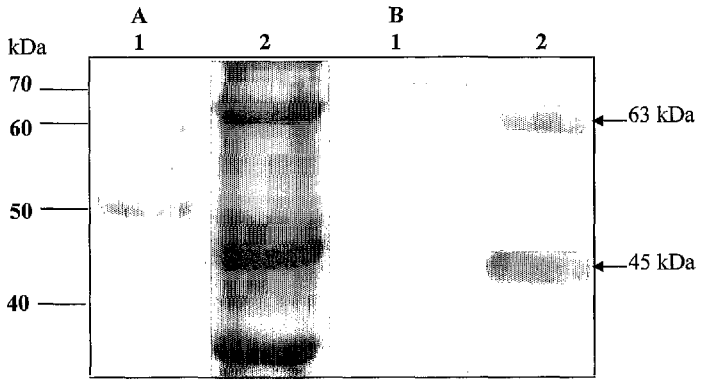

Figure 1

(57) Abstract: The present invention provides a method to enhance the recovery of recombinant N protein of Nipah virus produced in *Escherichia coli*, in which by identifying and inhibiting the specific proteases in the cell lysate are achieved by an integrated use of a bioinformatics tool and a protease inhibition experimental setup.

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A METHOD FOR CONTROLLING PROTEOLYTIC DEGRADATION OF RECOMBINANT PROTEINS

Field of the invention

The present invention relates to a method for controlling proteolytic degradation of recombinant nucleocapsid protein (NCp) of Nipah virus (NiV).

Background of the invention

Protein degradations caused by the endogenous proteolytic activity would reduce the yield of the expressed protein, and therefore, protease inhibitor cocktails and specific protease inhibitors are important tools for microbial proteomic studies. Most protease inhibitors interact with their target proteases by binding to its active site or modify an amino acid residue of the protease active site, resulting in the formation of a stable inactive protease inhibitor complex.

The NCp of NiV has been successfully produced in microbial system, and it is highly antigenic and immunogenic [see Ong *et al.* (2002), Biochem Mol Biol Biophys 6:347-50]. Recombinant protein of the NCp gene has potential to be developed as a diagnosis tool for NiV infection and to replace the high risk crude formalin-inactivated NiV antigen. The major problem during the production and purification of the NCp of NiV is the low recovery yield due to the proteolytic degradation. Hence a targeted strategy to inhibit the endogenous host proteolytic degradation recombinant NCp of NiV is presented in this invention.

Summary of the invention

The present invention is directed to a method to identify the specific endogenous proteases that cause the proteolytic degradation of the target recombinant protein (NCp) by adding the specific protease inhibitors to the buffer system. The proteolytic degradation activity was inhibited and as a result substantial improvement in the recovery of the recombinant NCp of NiV. The method of the present invention enables substantial improvement in recovery yield and requires short process time to

optimise the formulation of buffer system which is able to inhibit the proteolytic activity.

The present invention is directed to a method to identify the specific endogenous protease that causes the proteolytic degradation of a recombinant protein and inhibit its activity; the method comprises the steps of: (a) identifying the size of a degraded protein with an immunodetection method; (b) analysing amino acid sequence of the recombinant protein to identify the specific proteases that cause the degradation of the recombinant protein; (c) confirming the specific endogenous protease identified with immunodetection method; and (d) inhibiting proteolytic degradation of the recombinant protein with a specific protease with a specific protease inhibitor.

In the present embodiment, the inhibitor is selected from the group consisting of: phenylmethylsulphonyl fluoride (PMSF), 4-(2-aminoethyl) benzene sulfonyl fluoride hydrochloride (AEBSF) and aprotinin.

In one embodiment, the inhibitor is protease inhibitor is phenylmethylsulphonyl fluoride (PMSF).

In still another embodiment the concentration of phenylmethylsulphonyl fluoride (PMSF) comprises from 0.8 mM to 1.2 mM.

Brief description of the drawings

Figure 1: illustrates an example of the SDS-PAGE gel (A) and Western blot (B) of the NCp of NiV precipitated with ammonium sulphate.

Figure 2: illustrates an example of predicted serine cleavage site (underlined) of the NCp gene of NiV (SEQ.ID No.1) by the peptide cutter program of the preferred embodiment.

Figure 3: illustrates an example of the SDS-PAGE gel (A) and Western blot (B) for detecting the presence of potential protease in *E.coli* BL21(DE3).

Figure 4: illustrates an example of the result of the effect of addition of protease

inhibitors on the yield of NCp of NiV using Western blot analysis.

Figure 5: illustrates an example result of the dose dependence of the PMSF supplementation on the recovery yield of the NCp of NiV from *E.coli* lysate.

Detailed description of the preferred embodiment of the invention

Definitions

As used herein, the abbreviation "NCp of NiV" refers to the nucleocapsid protein of Nipah Virus.

As used herein, the abbreviation "NCp polypeptide" encompasses any polypeptide derived from the full length and naturally occurring NiV-NCp polypeptide listed herein as SEQ ID NO:1. The NiV-NCp polypeptide exemplified in this application is provided as example and do not limit the intent of this invention to include other polypeptide.

As used herein, "inhibits" means reducing, slowing or interfering with a process.

As used herein, the abbreviation "ClpP" refers to the caseinolytic peptidase.

The following polypeptides are useful in the present invention (a sequence listing according to ST.25 is also included in the application of the present invention):

SEQ ID NO: 1, amino acid sequence of full length mature protein of NCp of NiV

MSDIFEEAAS FRSYQSKLGR DGRASAATAT LTTKIRIFVP ATNSPELRWE LTLFALDVIR SPSAAESMKV GAAFTLISMY SERPGALIRS LLNDPDIEAV IIDVGSMVNG IPVMERRGDK AQEEMEGLMR ILKTARDSSK GKTPFVDSRA YGLRITDMST LVSAVITIEA QIWILIAKAV TAPDTAEESE TRRWAKYVQQ KRVNPFFALT QQWLTEMRNL LSQSLSVRKF MVEILIEVKK GGSAKGRAVE IISDIGNYVE ETGMAGFFAT IRFGLETRYP ALALNEFQSD LNTIKSLMLL YREIGPRAPY MVLLEESIQT KFAPGGYPLL WSFAMGVATT IDRSMGALNI NRGYLEPMYF RLGQKSARHH AGGIDQNMAN RLGLSSDQVA ELAAAVQETS AGRQESNVQA REAKFAAGGV LIGGSDQDID EGEEPIEQSG RQSVTFKREM

SISSLANSVP SSSVSTSGGT RLTNSLLNLR SRLAAKAAKE AASSNATDDP AISNRTQGES EKKNNQDLKP AQNDLDFVRA DV

SEQ ID NO: 2 - degraded polypeptide sequence
QESNVQAR

SEQ ID NO: 3 - degraded polypeptide sequence
AAAVQETSAGRQESNVQARE AKF

SEQ ID NO: 4 - degraded polypeptide sequence
RLGQKSARHHAGGIDQNMANRLGLSSDQVAELAAAVQETS AGRQESNVQAREAKF

SEQ ID NO: 5- degraded polypeptide sequence
GRQESNV

The inventors have utilized a bioinformatics tool to identify the proteolytic cleavage site and potential endogenous protease by analysing the amino acids sequence that deduced from the NCp gene of NiV, obtained from Gene Bank (Accession number: AJ564621). The potential endogenous protease that caused the cleavage of target peptide and its cleavage site from the amino acid sequence of NCp of NiV was successfully deduced. The prediction of the right protease that attacks the target protein and the supplementation of the specific inhibitor to the buffer system has substantially inhibited the activity of the endogenous proteases and improved the recovery yield of the recombinant NCp of NiV. This method improved the recovery yield substantially and it requires short process time to optimise the formulation of buffer system which is able to inhibit the proteolytic activity.

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention.

Figure 1 illustrates that two major protein bands were detected in the *E. coli* lysate by the swine anti-NiV serum: the expected 63 kDa full length NCp polypeptide and a much smaller 45 kDa NCp protein. This Western blot results confirm that this smaller protein band is the degraded form of the NCp polypeptide. A quantification analysis revealed that this smaller size NCp constitutes 70% of the total NCp expressed in *E. coli* BL21(DE3). (A) SDS-PAGE gel and (B) Western blot of the NCp of NiV precipitated with ammonium sulfate. Lane 1, molecular weight markers in kDa; Lane 2, NCp. Swine anti-NiV sera were used to detect the presence of recombinant NCp in *E. coli* cell lysate.

Figure 2 illustrates the predicted serine cleavage site (underlined) of the NCp gene of NiV by the bioinformatics program. The protease cleavage sites of the NCp of NiV (accession number AJ564621) of **SEQ ID NO: 1** are shown with the following symbols. ↓ : indicates arginine C proteinase, * : chymotrypsin (C-term to Phe/Tyr/Trp/Met/Leu, not before Pro), . : chymotrypsin (C-term to Phe/Tyr/Trp, not before Pro) and o : proteinase K. As the degraded NCp has a molecular mass about 45 kDa as illustrates in **Figure 1**, therefore it is predicted that a protease could cleave this protein at residue 404 in the N protein. The potential proteases that cleave the amino acid sequence around this position are listed in Table 2 and it is likely that the proteases that cleave position 404 are serine proteases.

Table 2: The protease cleavage peptide sites of NCp predicted by the PeptideCutter program

Possible protease	**cleavage site**	**Degraded peptide sequence**	**Suitable inhibitor**
Arginine C proteinase	404-411	Gln Glu Ser Asn Val Gln Ala Arg **(SEQ ID NO: 2)**	PMSF, AEBSF Hydrochloride, Aprotinin Bovine Lung
Chymotrypsin (C-term to	393-415	Ala Ala Ala Val Gln Glu Thr Ser Ala Gly	

F/Y/W/M/L, not before P)		Arg Gln Glu Ser Asn Val Gln Ala Arg Glu Ala Lys Phe **(SEQ ID NO: 3)**	
Chymotrypsin (C-term to F/Y/W, not before P)	361-415	Arg Leu Gly Gln Lys Ser Ala Arg His His Ala Gly Gly Ile Asp Gln Asn Met Ala Asn Arg Leu Gly Leu Ser Ser Asp Gln Val Ala Glu Leu Ala Ala Ala Val Gln Glu Thr Ser Ala Gly Arg Gln Glu Ser Asn Val Gln Ala Arg Glu Ala Lys Phe **(SEQ ID NO: 4)**	
Proteinase K	402-408	Gly Arg Gln Glu Ser Asn Val **(SEQ.ID.No.5)**	

Figure 3 illustrates the presence of the ClpP, a serine proteases, in the cell lysate. (A) SDS-PAGE gel and (B) Western blot for detecting the presence of potential proteases in *E. coli* BL21 (DE3). Lane 1, molecular weight markers in kDa; Lane 2, *E. coli* cell lysate. The Western blot (B) shows that the ClpP is indeed presence in the cell lysate and most probably responsible to the degradation of the N protein.

Figure 4 illustrates the effects of the addition of protease inhibitors on the yield of NCp using Western blot analysis. PMSF is the most effective inhibitor among all the serine protease inhibitors (PMSF, AEBSF and aprotinin). The recovery yield after adding PMSF was about 2-fold higher than the control (sample without any protease inhibitor supplementary). The yield of NCp treated with AEBSF and aprotinin was about 1.3 times higher than the control. The lysate sample was treated with each of the indicated protease inhibitors at the following concentrations: (1) none, (2) 1 mM PMSF, (3) 1 mM AEBSF, and (4) 1 mM aprotinin. Lane 1: the lysate was not treated with any protease inhibitor, which served as the control.

Figure 5 illustrates the result of the dose dependence of the PMSF supplementation on the recovery yield of the N protein of NiV from *E. coli* lysate. One mM of PMSF is the optimal concentration to inhibit the proteolytic activity of endogenous serine proteases. A further increment of PMSF concentration (1.2 mM) did not improve the recovery yield of the N protein.

The invention now being generally described, the same will be better understood by reference to the following detailed examples which are provided for purposes of illustration only and are not to be limiting of the invention unless so specified.

Example 1:

Prediction of the potential protease and cleavage site

The amino acid sequence of the NCp of NiV was obtained from the National Centre Biotechnology Information homepage, and was input into a peptide characterisation bioinformatics tool. There are several kinds of proteases present in the *E. coli* extract, which are capable of degrading the proteins. The potential proteases that may cause the degradation of NiV and their cleavage sites were identified by analysing the amino acid sequence of the protein.

Example 2:

Production of the recombinant NCp of NiV

E. coli strain BL21(DE3) harbouring plasmid pTrcHis$_2$ expressing the NCp was grown overnight in 1 L of Luria Bertani (LB) broth containing ampicillin (50 µg/ml) at 25°C, pH 7 with shaking. When the density of cell culture (OD_{600} reading) reached 0.6–0.8, protein expression was induced by adding of isopropylthio-β-D-galactoside (IPTG) to a final concentration of 1 mM and was further incubated for another 5 h at 25°C. The cells were harvested by centrifugation at 8000 x *g* (Avanti JLA-16.250, Beckman-Coulter, USA) for 20 min. Prior to the cell disruption, the cell pellet was washed with distilled water and centrifuged again at 8000 x *g* (Avanti JLA-16.250, Beckman-Coulter, USA) for 20 min at 4°C.

Example 3:

Recovering of NCp of NiV

E. coli cell pellet was resuspended in extraction buffer (25 mM HEPES buffer pH 8) supplemented with 0.2 µg/ml lysozyme and 4 mM $MgCl_2$. The initial crude cell homogenate sample was added immediately with specific protease inhibitors as listed in Table 1 that inhibit the potential protease and then subjected to sonication for 30 s with 60 s intervals in an ice bath for a duration of 50 min. The sonication was operated using an ultrasonic homogenizer (Labsonic U-Ultra 50, B. Braun, Germany) at 20 kHz equipped with a needle titanium probe (40 T) of 4 mm diameter and 127 mm length (Model 853 811/5). The cell lysate was clarified by centrifugation at 18,000 x *g*. The recombinant protein was precipitated with ammonium sulfate at 30% saturation. Then dialysis was carried out with Tris-NaCl buffer (50 mM Tris, 100 mM NaCl; pH 8.0). The dialysed protein solution was then assayed for total protein and NCp concentration.

Table 1: Preparation of the protease inhibitors stock

Inhibitor	Molecular weight	Stock concentration	Working concentration
PMSF	174.2	100 mM in dimethyl sulfoxide (DMSO)	1 mM
AEBSF Hydrochloride	239.5	400 mM in H_2O	1 mM
Aprotinin	6512	3 mM in H_2O	1 mM

Example 4

(A) The SDS-polyacrylamide gel (SDS-PAGE) analysis was performed under denaturing condition using a Mini-Protean 3 apparatus (Bio-Rad, USA). The reducing agents used in the electrophoresis buffer was 0.1% (w/v) SDS. Acrylamide of 12% (w/v) were used for resolving and stacking gels, respectively. After a 75-min electrophoresis at constant current of 16 mA, the gels were stained with Coomassie® Brilliant Blue (CBB) R-250 (ICI Ltd.) and destained with destaining solution containing 10% (v/v) methanol and 10% (v/v) acetic acid until a clear background was obtained.

(B) The released protein concentration was measured using the Bradford assay. Bovine-serum albumin (BSA) was used as standard and the sample was determined spectrophotometrically under a wavelength of 595 nm.

(C) Quantitation of NCp of NiV was determined by Western blots method Proteins were transferred to nitrocellulose membranes by using the Transblot SD® semidry transfer cell (Bio-Rad, USA), as described above. The NCp concentrations from various samples were estimated by comparing the intensity of the bands from Western blots with the help of an internal standard of the NCp purified using sucrose gradient ultracentrifugation. The bands were quantified by using the Volume Tools from the Bio-Rad imaging devices supported by the Quantity One® software (Bio-Rad). A volume is the intensity data inside a defined boundary drawn on the images. The intensity of the data inside the boundary and that of other objects can be compared using the Volume Analysis report.

The invention being thus described, it will be apparent that the same may be varied in many ways. Such variations are to be regarded as within the scope of the invention, and all such modifications as would be apparent to one skilled in the art are intended to be within the scope of the following claims.

Claims

1. A method to identify the specific endogenous protease that causes the proteolytic degradation of a recombinant protein and inhibit its activity, the method comprises the steps of:
    a) identifying the size of a degraded protein with an immunodetection method;
    b) analysing amino acid sequence of the recombinant protein to identify the specific proteases that cause the degradation of the recombinant protein;
    c) confirming the specific endogenous protease identified with immunodetection method; and
    d) inhibiting proteolytic degradation of the recombinant protein with a specific protease with a specific protease inhibitor.

2. The method according to claim 1, wherein said the recombinant protein is produced in recombinant cells.

3. The method according to claim 2, wherein the said recombinant cells is *Escherichia coli.*

4. The method according to claim 1, wherein said recombinant protein is recombinant N protein of NiV (NP-NiV) of SEQ ID NO: 1.

5. The method according to claim 4, wherein said NP-NiV is a full length 63 kDa protein version.

6. The method according to claim 1, wherein said immunodetection method is Western blot.

7. The method according to claim 4, wherein said degraded sequence protein of NP-NIV comprises peptide sequence set forth in any one of SEQ ID NOS:1-4.

8. The method according to claim 1, wherein said the analysis of amino acid sequence is performed with a bioinformatics tool.

9. The method according to claim 8, wherein said bioinformatics tool is used to predict potential endogenous proteases that cause the degradation of a recombinant protein.

10. The method according to claim 9, wherein said bioinformatics tool is used to predict the potential cleavage sites of the endogenous proteases on the recombinant protein.

11. The method according to claim 1, wherein said the specific protease identified is a serine protease.

12. The method according to claim 11, wherein said the serine protease identified is of chymotripsin type.

13. The method according to claim 12, wherein said chymotripsin type serine protease is caseinolytic peptidase (ClpP).

14. The method according to claim 1, wherein said inhibiting of proteolytic degradation is performed by the addition of protease inhibitors to the cell lysate.

15. The method according to claim 14 wherein said protease inhibitors is selected from the group consisting of phenylmethylsulphonyl fluoride (PMSF), 4-(2-aminoethyl) benzene sulfonyl fluoride hydrochloride (AEBSF) and aprotinin.

16. The method according to claim 14, wherein said protease inhibitor is phenylmethylsulphonyl fluoride (PMSF).

17. The method according to claim 16, wherein said the suitable concentration of phenylmethylsulphonyl fluoride (PMSF) is 0.8-1.2 mM.

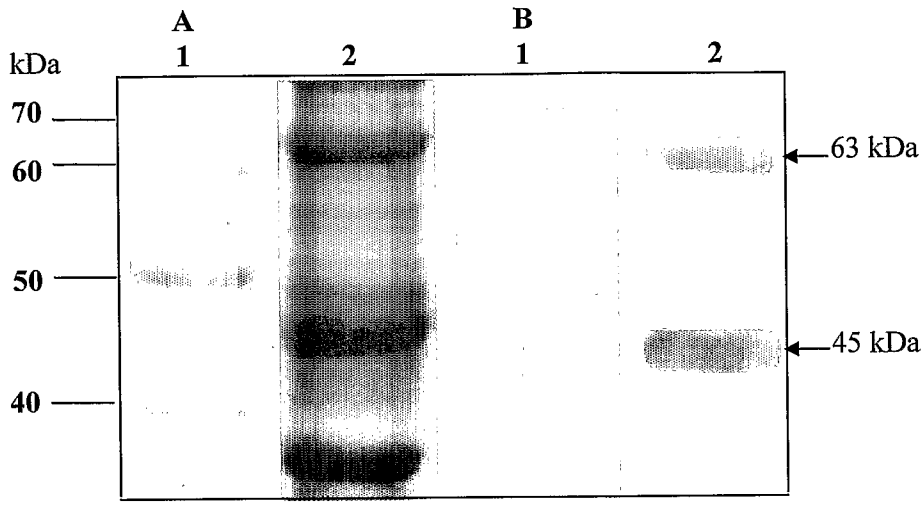
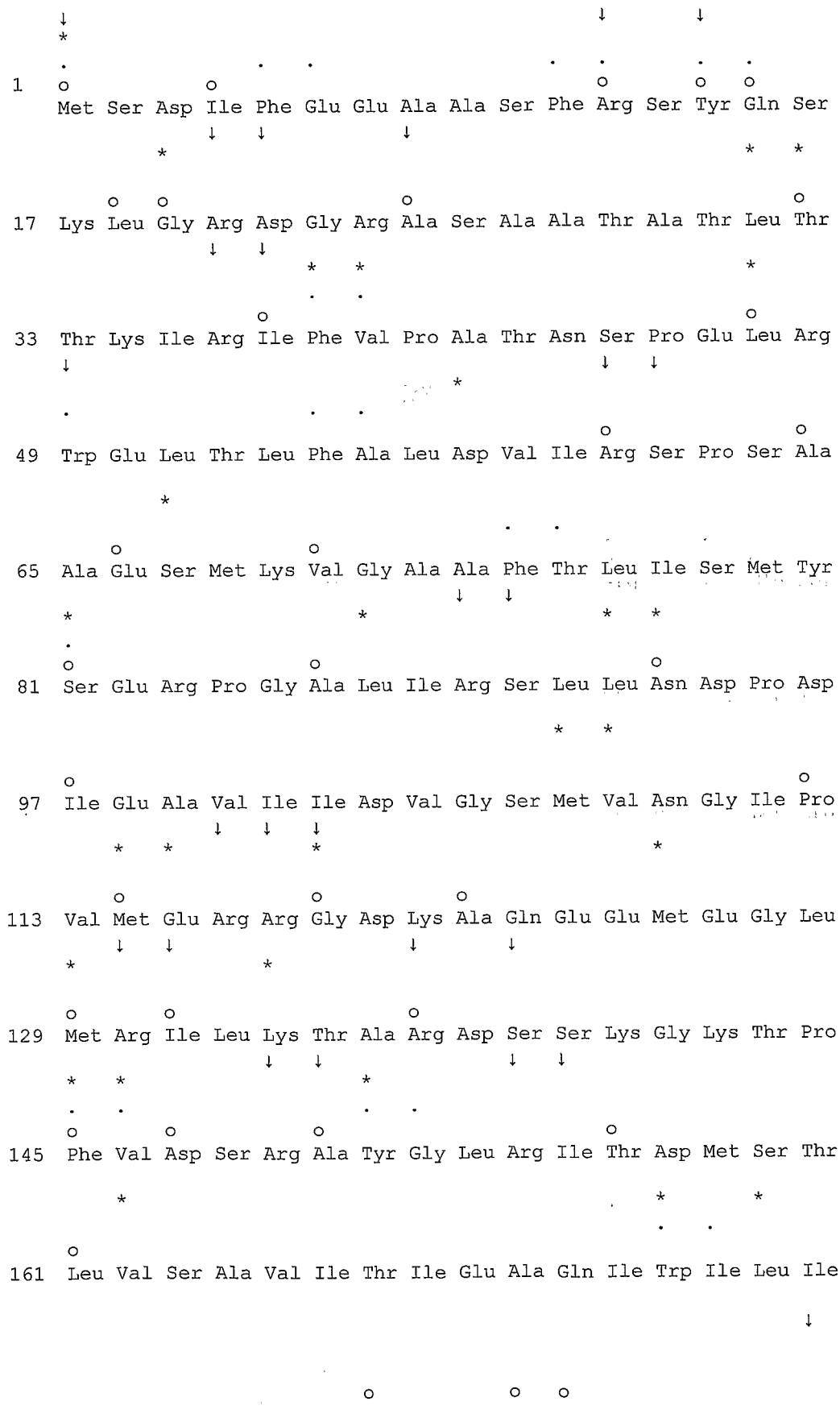


Figure 1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49 Trp Glu Leu Thr Leu Phe Ala Leu Asp Val Ile Arg Ser Pro Ser Ala
65 Ala Glu Ser Met Lys Val Gly Ala Ala Phe Thr Leu Ile Ser Met Tyr
81 Ser Glu Arg Pro Gly Ala Leu Ile Arg Ser Leu Leu Asn Asp Pro Asp
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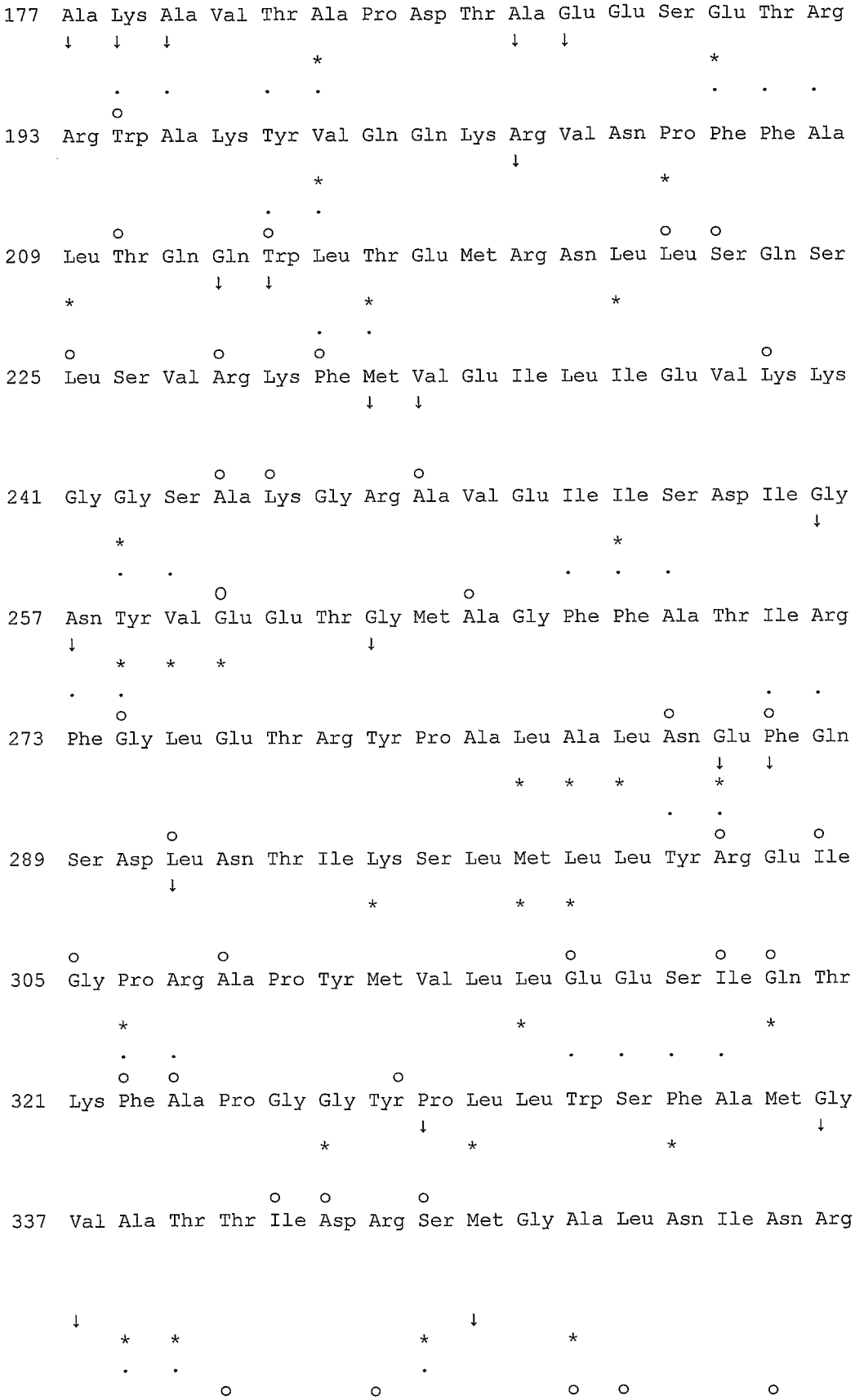
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257 Asn Tyr Val Glu Glu Thr Gly Met Ala Gly Phe Phe Ala Thr Ile Arg
273 Phe Gly Leu Glu Thr Arg Tyr Pro Ala Leu Ala Leu Asn Glu Phe Gln
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337 Val Ala Thr Thr Ile Asp Arg Ser Met Gly Ala Leu Asn Ile Asn Arg

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353  Gly Tyr Leu Glu Pro Met Tyr Phe Arg Leu Gly Gln Lys Ser Ala Arg
     ↓                                                ↓   ↓
                                                  *            *   *

                                                  o   o       o
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     *                           *   *
     o               o                                   o
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             ↓   ↓                           ↓                   *
                                                                 .   .
     o   o                       o
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                         *
                             o                           o   o
417  Ala Gly Gly Val Leu Ile Gly Gly Ser Asp Gln Asp Ile Asp Glu Gly
                                         ↓                       ↓
                                                                 *
                                                                 .   .
                     o   o                       o               o
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                                 *
                     o                           o               o
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                                 ↓                               ↓
                                 *                   *
     O                           o   o           o               o
465  Ser Thr Ser Gly Gly Thr Arg Leu Thr Asn Ser Leu Leu Asn Leu Arg
     ↓   ↓   ↓
                 *
             o
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                                     ↓   ↓

     o               o       o
497  Thr Asp Asp Pro Ala Ile Ser Asn Arg Thr Gln Gly Glu Ser Glu Lys
                         *   *                   *
                                                             .   .
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     ↓   ↓       ↓
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Figure 2

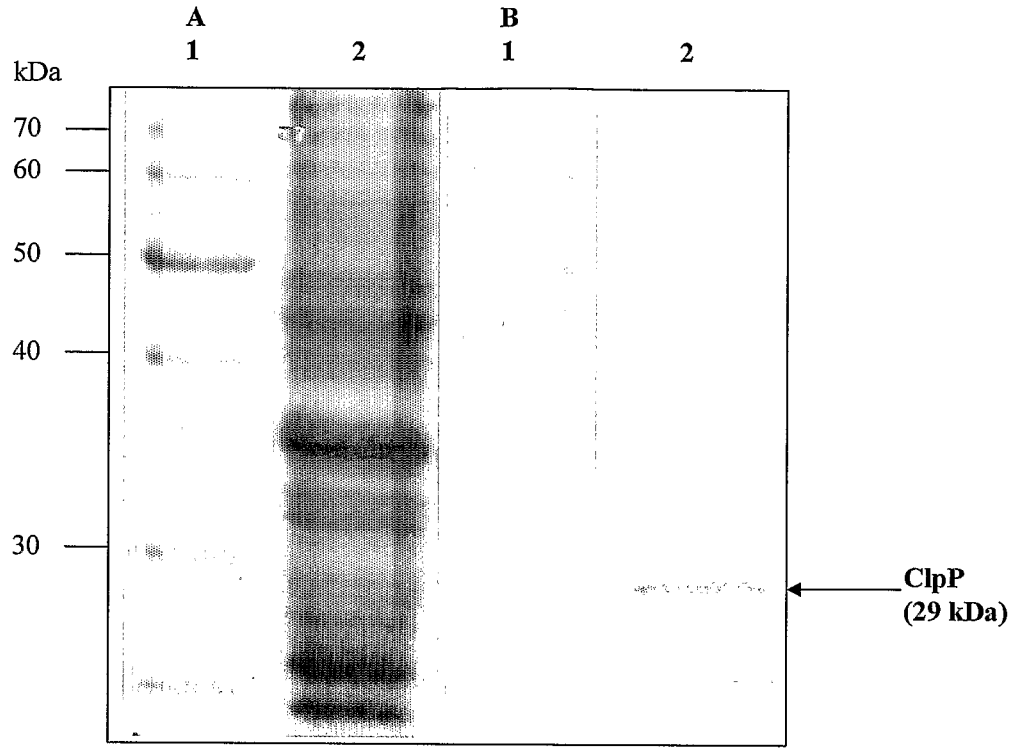
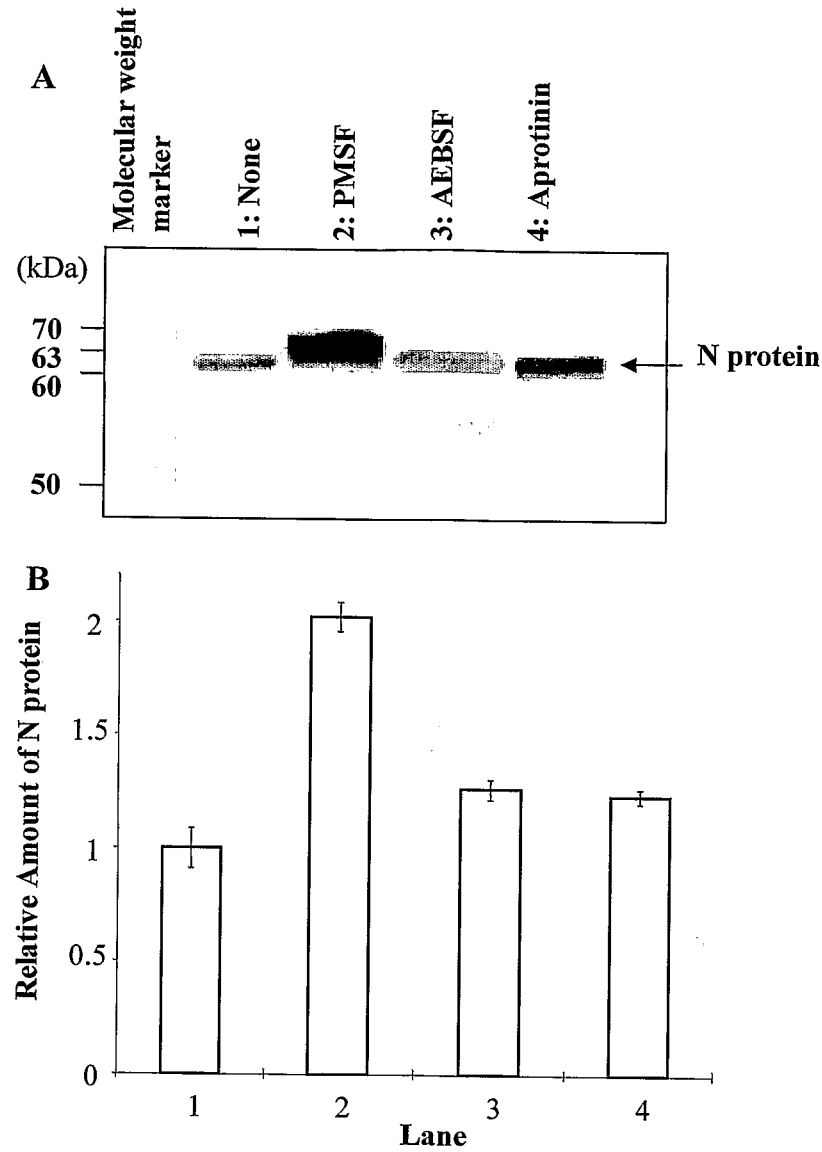


Figure 3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2: PMSF
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4: Aprotinin
(kDa)
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Relative Amount of N protein
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1
0.5
0
1
2
3
4
Lane

Figure 4

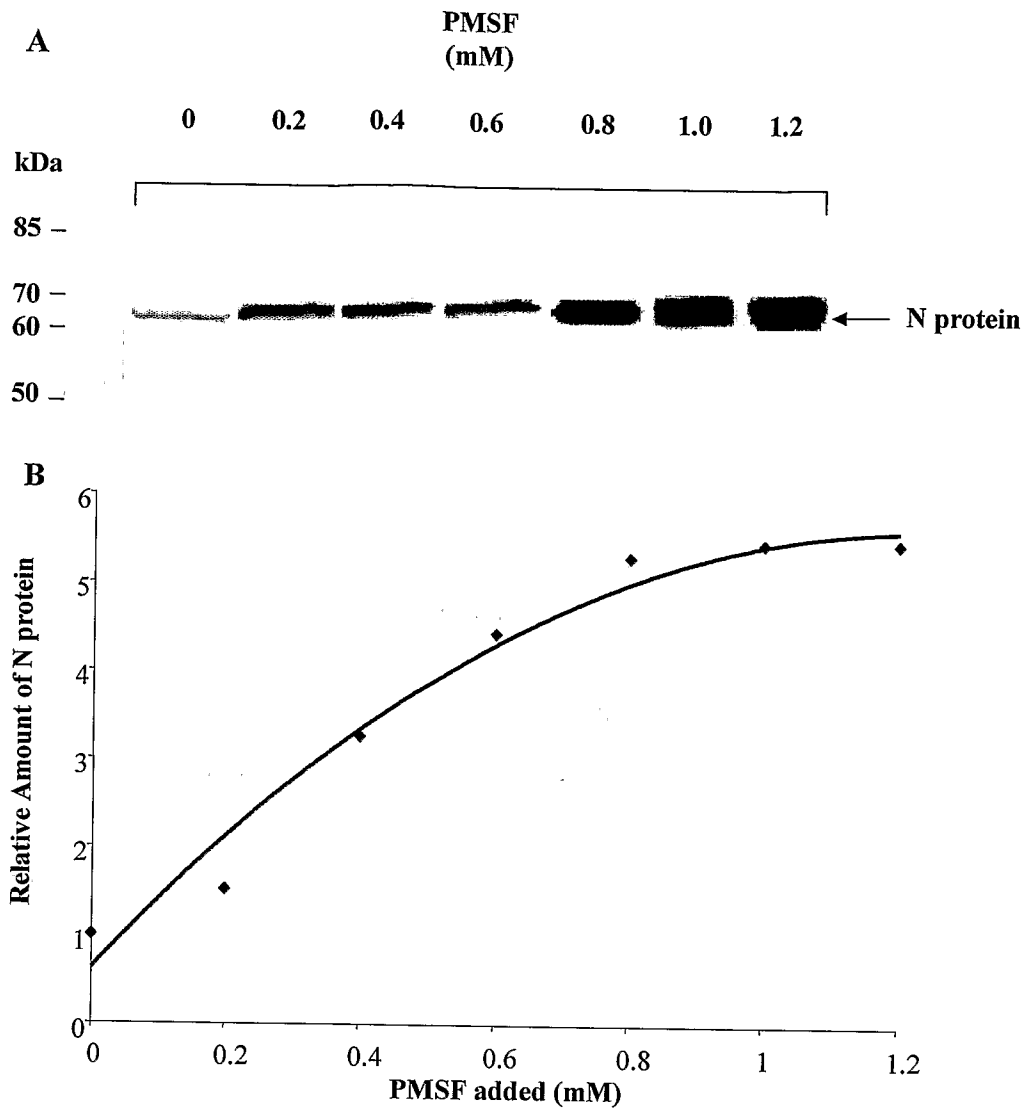
A
PMSF
(mM)
0
0.2
0.4
0.6
0.8
1.0
1.2
kDa
85 –
70 –
60 –
50 –
N protein
B
Relative Amount of N protein
0
1
2
3
4
5
6
0
0.2
0.4
0.6
0.8
1
1.2
PMSF added (mM)

Figure 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/MY2009/000105

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/017293 A2 (DIGILAB BIOVISION GMBH [DE]; BUDDE PETRA [DE]; SCHULZ-KNAPPE PETER [DE) 15 February 2007 (2007-02-15)	1-2
Y	table 1 example 8	3-17

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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3 December 2009	11/12/2009

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Jones, Laura

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INTERNATIONAL SEARCH REPORT

International application No
PCT/MY2009/000105

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	DIEDERICH S ET AL: "Role of endocytosis and cathepsin-mediated activation in Nipah virus entry" VIROLOGY, ACADEMIC PRESS,ORLANDO, US, vol. 375, no. 2, 5 June 2008 (2008-06-05), pages 391-400, XP022665850 ISSN: 0042-6822 [retrieved on 2008-03-14] the whole document	1-17
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/MY2009/000105

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