CHAPTER 3

ATTEMPTS TOWARDS CRYSTALLIZATION OF DEN-2 PROTEASE COMPLEX

3.1 Protein crystallization of DEN-2 NS2B-NS3pro

Until very recently, attempts to crystallize the protease complex that is responsible for viral replication of DEN-2 by various groups have not been successful. Crystallization of DEN-2 protease complex was inevitable as structural information is greatly required to enable us to perform structural studies involving compounds with inhibitory activities in our effort to search for a therapeutic agent against dengue infections. Understanding the structural interactions between protease and ligand will provide important information for inhibitory activity studies which may lead to a design of candidate as anti-dengue drug.

This chapter discusses the experiments performed in attempts to crystallize the DEN-2 NS2B-NS3pro complex. The data and information obtained from a crystal structure will be used as input for the subsequent parts of the project involving computational modelling studies. Unfortunately, our attempts did not produce any protein crystals. The reasons behind these unsuccessful attempts are established and will be discussed in this chapter.

3.2 Materials and methods

3.2.1 Materials

3.2.1.1 Materials and instruments for protein overexpression and purification

Overexpression clone of *Escherichia coli* (strain XL1-Blue MRF) containing NS2Bco-NS3pro fragment in pQE-30 vector was obtained from Prof. Padmanabhan (previously at the University Kansas, U.S.A). Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Calbiochem. Ni^{2+}-nitrilotriacetic acid (NTA)-agarose resin

was from Qiagen (Chadworth, CA). Fluorogenic peptide subtrate Boc-Gly-Arg-Arg-MCA was obtained from Peptide Institute, Inc. (Osaka, Japan). For column separation, Sephadex G-75 was supplied by Amersham Pharmacia and Hiload 16/60 Superdex 75 preparative grade. Bio-Rad Protein Assay kit (Bio-Rad, U.S.A.) was used for determination of soluble protein concentration. Pre-stained SDS-PAGE standards (Low range) from BioRad and Mark12^{TM} Unstained Standard (Invitrogen Corp.) were used as standard in SDS-PAGE for protein molecular weight separation.

Sorvall GSA and Sorvall RC-5B refrigerated superspeed centrifuges were used for cell sedimentation. Tomy autoclave SS-325 was used for autoclaving solutions and glasswares. For protease bioassays, the Cary Eclipse Fluorescence spectrophotometer (Varians) was used to measure the intensity of the fluorogenic moiety (7-amino-4-methylcourmarin, AMC) from the cleaved fluorogenic peptide substrate (excitation at 385 nm and emission at 465 nm). Shidmadzu UV-Visible Recorder Spectrophotometer (UV-160) was used for optical density (OD) and quantitative protein assay.

3.2.1.2 Media for bacterial cell growth

Luria-Bertani (LB) agar

Formulation: 0.5 g Yeast extracts, 1.0 g bacto-tryptone, 1.0 g NaCl, 1.5 g agar, 100 ml distilled water, 0.1 ml ampicillin (100 mg/ml).

The ingredients above were dissolved in 75 ml distilled water. The solution was adjusted to pH 7.0 and brought to a final volume of 100 ml. The medium was then sterilized by autoclaving for 20 minutes at 121^{o}C. When the medium reached 45^{o}C, it

was supplemented with ampicillin (working concentration of 100 µg/ml). 25 ml of the medium was poured into 4 LB plates. The plates were dried, wrapped and stored at 4^{o}C.

Luria-Bertani (LB) medium

Formulation: 5 g Yeast extracts; 10 g bacto-tryptone; 10 g NaCl; 1000 ml distilled water; 1 ml ampicillin (100 mg/ml).

The ingredients above were dissolved in 900 ml distilled water. The solution was adjusted to pH 7.0 and brought to a final volume of 1000 ml. The medium was then sterilized by autoclaving for 20 minutes at 121^{o}C. The medium was cooled to room temperature and supplemented with ampicillin (working concentration of 100 µg/ml). The medium was wrapped and stored at 4^{o}C.

3.2.1.3 Stock solutions

Ampicillin stock solution (100 mg/ml)

The stock solution was prepared in deionised water and filtered through 0.22 µm pore size cellulose acetate membrane (Sartorius). The solution was then dispensed into aliquots of 1 ml and stored at 4^{o}C.

IPTG stock solution (0.5 M)

1.192 g of IPTG was dissolved in 10 ml deionised water (dH_2O) and filtered. The solution was then dispensed into aliquots of 1 ml and stored at -70^{o}C.

Imidazole stock solution (5 M)

3.40 g imidazole was dissolved in deionised water and made up to a volume of 10 ml.

3.2.1.4 Buffers for protein purification and dialysis

Buffer A 100 mM Tris-HCl, 300 mM NaCl, pH 8.0

Formulation: 2.42 g Tris; 3.51 g NaCl; 200 ml dH_2O (deionised water).

The ingredients above were dissolved in 150 ml dH_2O. The solution was adjusted to pH 8.0 with HCl and the volume was made up to 200 ml with dH_2O.

Buffer B 100 mM Tris-HCl, 300 mM NaCl, 6 M Urea, pH 8.0

Formulation: 6.05 g Tris; 8.78 g NaCl; 180.18 g urea; 500 ml dH_2O.

The ingredients above were dissolved in 450 ml dH_2O. The solution was adjusted to pH 8.0 with HCl and the volume was made up to 500 ml with dH_2O.

Buffer C 100 mM Tris-HCl, 300 mM NaCl, 6 M Urea, pH 7.5

Formulation: 2.42 g Tris; 3.51 g NaCl; 72.07 g urea; 200 ml dH_2O (deionised water).

The ingredients above were dissolved in 150 ml dH_2O. The solution was adjusted to pH 7.5 with HCl and the volume was made up to 200 ml with dH_2O.

Buffer D 100 mM Tris-HCl, 300 mM NaCl, pH 7.5

Formulation: 12.1 g Tris; 17.55 g NaCl; 1000 ml dH_2O.

The ingredients above were dissolved in 900 ml dH_2O. The solution was adjusted to pH 7.5 with HCl and the volume was made up to 1000 ml with dH_2O.

3.2.1.5 Solutions for Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)

30 % Acrylamide/ 0.8 % Bisacrylamide (w/w) solution

Formulation: 60 g Acrylamide; 1.6 g N,N'-methylene bisacrylamide.

The acrylamide was firstly dissolved in 150 ml distilled water. Then, the bisacrylamide was added into the solution and mixed until dissolved. The solution was brought to a final volume of 200 ml, filtered and stored at 4^{o}C in a tightly capped amber bottle.

4X, 1.5 M Tris.Cl / 0.4 % SDS, pH 8.8

Formulation: 91 g Tris base; 2 g SDS.

Tris was dissolved in 450 ml distilled water. The solution was adjusted to pH 8.8 with concentrated HCl and made up to a final volume of 500 ml with distilled water, filtered through a 0.45 μm filter and stored at 4^{o}C.

4X, 0.5 M Tris.Cl / 0.4 % SDS, pH 6.8

Formulation: 6.05 g Tris base; 0.4 g SDS.

Tris was dissolved in 50 ml distilled water. The solution was adjusted to pH 6.8 with concentrated HCl and made up to a final volume of 100 ml with distilled water,

filtered through a 0.45 μm filter and stored at 4^{o}C.

10 % w/v Ammonium persulfate (APS)

0.1 g Ammonium persulphate was dissolved in 1 ml distilled water in a 1.5 ml eppendorf tube. The solution was freshly prepared prior to use.

5X, Running buffer, SDS/ Electrophoresis buffer (concentrated stock)

Formulation: 7.55 g Tris base; 36.0 g glycine; 2.5 g SDS; 500 ml distilled dH_2O.

The ingredients above were mixed, stirred until dissolve and stored at 4^{o}C. This concentrated stock solution was diluted to 1X, SDS/ electrophoresis running buffer prior to use.

2X, SDS / sample loading buffer (concentrated stock)

Formulation: 2.5 ml 4X, 0.5 M Tris.Cl / 0.4 % SDS, pH 6.8 ; 2.0 ml glycerol ; 0.4 g SDS; 0.2 ml β-mercaptoethanol; 10.0 mg bromophenol blue.

The ingredients above were dissolved in distilled water and brought to a final volume of 10 ml. The sample dye solution was then aliquot into 1 ml volume size and stored at –70^{o}C.

Coomasie Blue staining solution

Formulation: 50 % (v/v) Methanol; 0.05 % (w/v) Coomasie Brilliant Blue R-250; 10 % (v/v) acetic acid; 40 % distilled dH_2O.

The Coomasie Brilliant Blue R-250 was dissolved in methanol before adding

acetic acid and water. The solution could be stored on open shelves at room temperature for six months. If precipitation occurred, the solution was filtered through Whatman filter paper to obtain a homogenous solution.

Destaining solution

Formulation: 7 % (v/v) Acetic acid; 5 % (v/v) methanol; 88 % (v/v) distilled dH_2O.

The solution was prepared in volume size of 1 litre.

Fixing solution

Formulation: 50 % (v/v) Methanol; 10 % (v/v) acetic acid; 40 % (v/v) distilled dH_2O.

The solution was prepared in volume size of 1 litre and could be stored on open shelves at room temperature for 1 month.

Gel drying solution (Promega Gel Drying Kit)

Formulation: 40 % (v/v) Methanol; 10 % (v/v) glycerol; 7.5 % (v/v) acetic acid; 42.5 % (v/v) distilled dH_2O.

The solution was prepared in volume size of 100 ml, mixed well and stored on open shelves.

3.2.1.6 12 % SDS-PAGE gel

A. Separating gel solution

Formulation: 6.00 ml 30 % Acrylamide/0.8 % bisacrylamide (w/w); 3.75 ml 4X, 1.5 M

Tris.Cl / 0.4% SDS, pH 8.8; 5.25 ml distilled dH_2O; 50 µl 10 % APS; 10 µl N,N,N',N'-tetramethylethyldiamine (TEMED).

A glass-plate sandwich was assembled using two clean glass plates and two 0.75 mm spacers, and was locked to a casting stand. The above ingredients were prepared in a 30 ml tube, swirled gently to mix properly. As soon as APS and TEMED were added and mixed properly, the solution was immediately poured into the pre-packed sandwich SDS-PAGE slab. Care was taken to avoid inclusion of bubbles. A layer of methanol (approximately 1 cm in height) was laid on top of the solution to compress the solution, ensuring an even separating gel formation. The gel was allowed to polymerize for 30 to 60 minutes at room temperature.

B. Stacking gel solution

Formulation: 650 µl 30 % Acrylamide / 0.8 % bisacrylamide (w/w); 1.25 ml 4X, 1.5 M Tris.Cl / 0.4 % SDS, pH 6.8; 3.05 ml distilled dH_2O; 25 µl 10 % APS; 5 µl TEMED.

The above ingredients were prepared in a 30 ml tube and swirled gently to mix properly. The methanol layer on top of the separating gel was poured out. The stacking solution was immediately poured into the sandwich gel slab, on top of the polymerized separating gel. A 0.75 mm Teflon comb was inserted into the layer of stacking gel solution. The stacking gel was allowed to polymerize for 30 to 45 minutes at room temperature.

3.2.1.7 Dialysis tubing preparation

Formulation: 20 g Sodium bicarbonate; 2 ml 0.5 M ethylenediaminetetraacetic acid (EDTA); 1000 ml dH_2O.

The ingredients above were mixed to form 2 % sodium bicarbonate / 1 mM EDTA (pH 8.0) solution. Dialysis tubing was cut into lengths of 18 to 24 inches and curled into a large volume of the above solution. The tubings were boiled for 10 minutes, rinsed twice with distilled water, and boiled again for 10 minutes in a large volume of 1 mM EDTA (pH 8.0). The tubings were allowed to cool and stored at 4^oC in a fresh solution of 1 mM EDTA (pH 8.0) in sterile distilled water (the tubings were submerged in the solution to enhance the storage condition). The tubings were washed thoroughly inside and outside with distilled water prior to use.

3.2.1.8 Precipitating buffers for crystallization trials

The following crystallization kits were used for crystallization screens:

Crystal screen™, Crystal screen 2™ and PEG / Ion Screen™ from Hampton Research, U.S.A; The Classics, The Pegs, The pHClear™, The SM1™, The $AmSO_4$ and The MPDs from Nextal Biotech; Wizard™ I and Wizard™ II from Emerald Biosystems, U.S.A.

The different formulations for each kit are listed in Appendix 1.

3.2.1.9 Cleaning and siliconizing cover slips

Microscope cover slips were repeatedly cleaned with detergent and rinsed with water. They were then dried in a drying cabinet. Siliconizing of the cover slips was carried out in a fume hood. A siliconizing solution (dimethyldicholrosilane solution; about 2 % in 1,1,1-tricholroethane) was poured into a small beaker and placed in a dessicator. The dried cover slips were placed into a glass plate and put on top of the beaker containing the siliconizing solution in the dessicator, which was then connected to a vacuum pump. The set-up was left under vacuum for about 30 minutes. The cover slips, coated with the siliconizing solution, were then cleaned with a fibre-free cloth, blown with blow dryer and stored in a dry container until used.

3.2.2 Methods

The protocols used in this study in the attempts to crystallize the DEN-2 protease are as illustrated in a flowchart in Figure 3.1.

3.2.2.1 Starter scale culture and glycerol stock preparation

A glycerol stock of *Escherichia coli* strain XL1-Blue MRF' transformed with the 6x-His-NS2B-3pro expression plasmid was obtained through personal contact with Yusof *et al.* (2000). An inoculum was streaked across one side of an LB agar plate using sterile technique. This step was repeated across the surface of the plate and the plate was incubated at 37^{o}C until colonies appear.

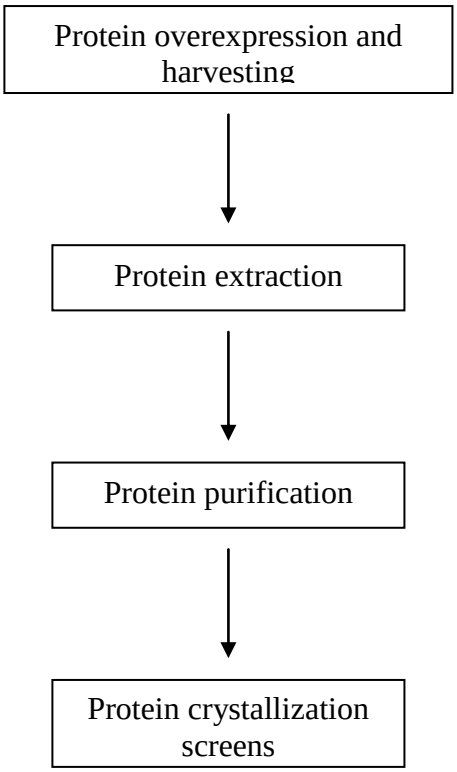


Figure 3.1 Flowchart of protocols involved in this study towards the crystallization trials of DEN-2 protease.

A single bacterial colony was isolated from an overnight LB agar plate and inoculated into a sterile culture tube containing 10 ml of LB broth. This procedure was repeated to yield several tubes of the bacterial mini-cultures. The tubes were loosely capped and grown at 37^{o}C for overnight in a shaker (Innova 4000 Incubator Shaker, New Brunswick Scientific). These stocks were used for larger scale culture and storage purposes. For storage purpose, 2.5 ml sterilized glycerol was added to each of the 10 ml culture (yielding a stock solution containing 20 % glycerol) and mixed well. One ml of the stock solution was aliquoted into 1.5 ml centrifuge tubes and stored at –70^{o}C.

3.2.2.2 Large scale protein overexpression, harvesting and extraction

Frozen glycerol stock was thawed on ice and 125 µl stock solution was transferred into an LB medium (50 ml) containing 100 µg/ml ampicillin. The culture was left to grow overnight at 37^{o}C, shaking at 250 rpm.

After 1 day, 10 ml of the overnight culture was inoculated into each of 6 x 1 litre LB medium containing ampicillin (100 µg/ml). Starting OD_{600} (optical density at 600 nm) of each medium was approximately 0.06. The cultures were incubated at 37^{o}C, with shaking at 200 rpm until OD_{600} reached approximately 0.6. The bacterial cells were induced for protein expression by addition of IPTG (0.5 mM working concentration) for 3 hours. The cells were harvested by ultra-centrifugation at 6 000 rpm (Sorvall RC-5B Refrigerated Superspeed centrifuge) for 15 minutes at 4^{o}C, and the pellets obtained were stored at –70^{o}C until used.

For protein extraction, the cell pellets were thawed on ice, resuspended in buffer A (100mM Tris.HCl, 300mM NaCl, pH 8.0) containing 1 mg/ml lysozyme and

incubated at 4^{o}C for 30 minutes. The solution was then centrifuged at 15 800 rpm for 1 hour at 4^{o}C. The supernatant was discarded and pellet was again resuspended under denaturing condition with buffer B (6M Urea, 100mM Tris.HCl, 300mM NaCl, pH 8.0), followed by cell lysis with sonication on ice (9 x 10 sec), and the lysate was kept on ice for 1 hour. The lysate was clarified by centrifugation at 15 800 rpm for 1 hour at 4^{o}C. The 6x-His-NS2B-3pro was predominantly soluble in the supernatant in its denatured form.

3.2.2.3 Protein purification

The supernatant containing the soluble, denatured protein was filtered through a 0.45 µm filter, batch-bound to 2 ml Ni^{2+}-NTA (nickel-nitrilotriacetic acid) resin (pre-equilibrated with buffer B) and incubated overnight at 4^{o}C. The resin was centrifuged briefly at 1000 x g, the unbound fraction collected, and the resin with bound protein was loaded onto a column (Bio-Rad; 1 x 3 cm). The column was washed extensively with 40 ml of buffer B. Protein elution was done by loading 10 ml buffer C (6 M Urea, 100 mM Tris.HCl, 300 mM NaCl, pH 7.5) containing 500 mM imidazole. The eluted fractions were collected in 0.5 ml volume size and analysed with 12 % SDS-PAGE. The elution profile was monitored using the Bio-Rad protein assay kit. Peak fractions were then pooled and subjected to gel filtration chromatography.

The next step of protein purification involved two types of column used in gel filtration chromatography: preparative Sephadex^{TM} G-75 column and pre-packed Hiload Superdex^{TM} 200 column. For the first type of column, a Bio-Rad column (50 cm x 1.5 cm) was used and loaded with pre-treated Sephadex^{TM} G-75 (bed volume was approximately 81 cm^3). Pre-treatment of the resin was done as follows: 5 g of

Sephadex^{TM} G-75 was soaked in 70 ml sterilized dH_2O. After discarding the supernatant, the resin was then equilibrated twice with buffer B. Sodium azide was then added (end concentration of 0.02 %) to act as anti-microbial, and the slurry was carefully poured into the column and packed to remove air bubbles.

The pooled peak fractions from the previous batch purification on nickel column were slowly loaded into the gel filtration column and separated (at 4^oC) under denaturing condition with buffer B at a constant flow rate of 1 ml min^{-1}. Eluted fractions were collected in 1.5 ml volume size and analysed with 12 % SDS-PAGE. The elution profile was monitored using the Bio-Rad protein assay kit.

Peak fractions with the desired molecular weight were pooled and diluted to less than 0.5 mg/ml in buffer B. Refolding of the denatured protein was performed by dialysis against four changes of buffer D (100 mM Tris.HCl, 300 mM NaCl, pH 7.5) for 48 hours at 4^oC. The dialysate was clarified by centrifugation (12 000 rpm for 1 hour) at 4^oC and concentrated using Vivaspin (MWCO 10 000) by centrifugation at 3000 x g for a few hours (Allegra X-12R centrifuge) until the volume was reduced to less than 2 ml.

The refolded protein solution was then subjected to gel filtration chromatography (pre-packed Hiload Superdex^{TM} 200 column) under native condition using buffer D to further purify the desired protease. The same separating conditions were used as previously described. The elution profile was monitored using the Bio-Rad protein assay kit and analysed with 12 % SDS-PAGE. Peak fractions of the purified protein were pooled and concentrated using Vivaspin by centrifugation until the concentration reached approximately 10 mg/ml.

3.2.2.4 Bio-Rad Protein assay

The Bio-Rad Protein assay, based on method of Bradford, is a simple and accurate procedure for determining concentration of solubilized protein. The dye reagent was prepared by diluting 1 part of the Dye Reagent Concentrate with 4 parts distilled dH_2O and filtered through Whatman #1 filter to remove particulates. Fifty microlitre of the protein solution was mixed with 2.5 ml of the diluted reagent, vortexed and incubated at room temperature for at least 5 minutes. Absorbance was measured at 595 nm. The concentration of protein after purification was estimated from the bovine serum albumin (BSA) standard curve.

3.2.2.5 Bovine serum albumin (BSA) standard curve

A stock of BSA solution with concentration 1 mg/ml was prepared in distilled dH_2O. Standard solutions of BSA ranging from 0.2 to 0.9 mg/ml were then prepared to construct a standard curve of absorbance at 595 nm.

3.2.2.6 7-Amino-4-methylcoumarin (AMC) standard plot for protein assay

An AMC standard plot was used in the determination of the NS2B-NS3 protease activity. A working stock of AMC with concentration 10 μM was prepared and standard AMC solutions ranging from 0 to 5.0 μM were made up.

3.2.2.7 Preparation of fluorogenic peptide substrate

Fluorogenic substrate, BOC-Gly-Arg-arg-MCA, (5.4 mg) was dissolved in 830 µl DMSO to furnish a concentrated stock solution of 10 mM. This stock was further diluted to 1 mM and 2.5 mM stock solutions. All the prepared stock solutions were stored at –70^{o}C in the dark to prolong the substrate stability.

3.2.2.8 Determination of protease kinetic properties

Protease activity (kinetic properties) was determined after its purification to ensure that the protease that goes through crystallization screens has activity. Protocols for protease assay follows those of Yusof *et al*. (2000) and Teoh (2004).

The reaction mixture constituted of a constant concentration of 1 µM of protease, substrate concentrations between 20 to 200 µM and Tris buffer. The end volume of each mixture was 200 µl. The activity of the protease complex was characterized by the fluorogenic substrate, which released the fluorogenic moiety (7-amino-4-methyl coumarin, AMC) upon cleavage by the active protease. The assays were incubated at 37^{o}C for 30 minutes. Measurement of the intensity of the released AMC was detected with Cary Eclipse Fluorescence spectrophotometer (Varians) at excitation wavelength of 385 nm and emission wavelength of 465 nm. The control (in the absent of substrate) and reaction assays were done in triplicate.

3.2.2.9 Crystallization screens

Most of the crystallization trials were carried out at the Department of Molecular

Biology and Biotechnology, the University of Sheffield, under the supervision of Prof. Dr. David Rice. In this study, the vapour-diffusion methods were employed in the crystallization trials involving the hanging drop and sitting drop methods. Formulations of the precipitating buffers are listed in Appendix 1. The concentration of the protein solution used in the screens was about 10 mg/ml. The protein solution was centrifuged at 13 000 rpm for 2 minutes to settle down any precipitant that might be present.

3.2.2.9(a) Hanging drop method

The hanging drop method was carried out manually using 24-well Linbro plates (Hampton Research, USA) (Figure 3.2a). An oil sealant ring was put around each well which was then filled with and 500 µl of the precipitating buffers. The protein solution was dropped onto a siliconized cover slip and an amount of the precipitating buffer from the well was added to it in the following volumes:

1 µl protein solution + 1 µl precipitating buffer; or

2 µl protein solution + 2 µl precipitating buffer.

The cover slip was then inverted over the well containing the precipitating buffer such that the drop hanged from its underside. Care was taken to ensure that the well was properly sealed to ensure that the drop gradually become dehydrated and equilibrated with the solution in the reservoir. This procedure was repeated for all the precipitating conditions. The plates were then incubated at 17^{o}C. The screens were monitored for any crystal formation by observation under the microscope.

(a)

(b)

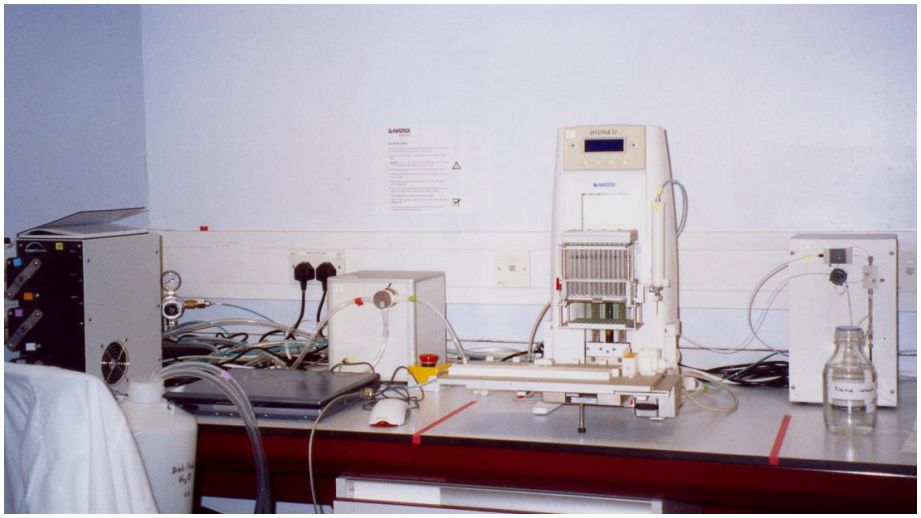


Figure 3.2 Diagram showing (a) a 24-well Linbro plate containing protein drops in sealed environment in the hanging drop method, and (b) the robotic system, The Hydra II-Plus-One System, available at the Department of Molecular Biology and Biotechnology, The University of Sheffield, used in the sitting drop method.

3.2.2.9(b) Sitting drop method

The sitting drop technique was performed by employing the automated system using the Hydra II-Plus-One System (Figure 3.2b). A drop of 0.2 µl protein solution and 0.2 µl precipitant was placed on each drop support of a 96 well Crystalquick plate (Greiner Bio-one, Starlab GMBH, Germany). Each reservoir contained 100 µl of precipitating buffer. The plate was sealed with microplate sealing film (Starseal Advanced Polyefin film, Starlab GMBH, Germany) and incubated at 17^{o}C. The screens were monitored for any crystal formation by observation under the microscope.

3.3 Results

3.3.1 Expression and purification of active DEN-2 NS2B-NS3pro

The cultures of competent *Escherichia coli* strain XL1-Blue MRF' transformed with the 6x-His-NS2B-NS3pro expression plasmid was obtained from Prof. Rohana Yusof, Department of Molecular Medicine, University of Malaya (personal communication). To express the DEN-2 protease complex, similar strategy, with minor modifications, as described by Yusof *et al.* (2000), was employed. Large scale overexpression of protein was performed to yield sufficient amount of purified protein for crystallization screens. Since the protein precursor was associated with insoluble inclusion bodies, it was solubilized from the insoluble pellet fraction of the bacterial cell lysate using a buffer containing urea (buffer B).

The protein precursor was put through several steps of purification. Firstly, purification was done using Ni^{2+} affinity column (as described in section 3.2.2.3). The

elution profile is as shown on Figure 3.3. The precursor was eluted with 500 mM imidazole (Figure 3.4, lane 3). The concentration of imidazole used seemed to be high, which may explain the co-elution of other proteins with lower molecular weight observed. Hence, gradient elution of the protein precursor was done using increasing concentrations of imidazole in the elution buffer to obtain cleaner protein precursor. From Figure 3.5, the precursor was observed to be eluted out of the solution with imidazole concentration as low as 2 mM. However, when the eluted protein solution was subsequently dialysed, the protein precursor did not refold into its native conformation. This is shown in Figure 3.6, lanes 4, 5 and 6. Consequently, buffer C containing 500 mM imidazole was still used to elute the protein precursor out of the Ni^{2+} affinity column.

The second purification step involved the use of size exclusion gel chromatography (as described in section 3.2.2.3). Initially, preparative Sephadex^{TM} G-75 was used (Figure 3.7). Later on, however, purification was done on pre-packed Hiload Superdex^{TM} 200 column, since this column resulted in better separation of proteins under denaturating condition (Figure 3.8). Fractions 11 to 14, contained the purified NS2Bco-NS3pro and thus were pooled together. The protein refolded into its active conformation when subjected to dialysis under native condition (Figure 3.4, lane 6). Unfortunately, as soon as the active conformation was obtained, auto-cleavage took place, releasing NS2B (~ 8 kDa), NS3pro (~ 23 kDa) and other degenerative products.

The following purification step involved passing the dialysate through the pre-packed Hiload Superdex^{TM} 200 column under native conditions. As shown in Figure 3.9, two peaks were observed in the polarograph, each representing the refolded protease. The peak on the right represented fractions which contained the active

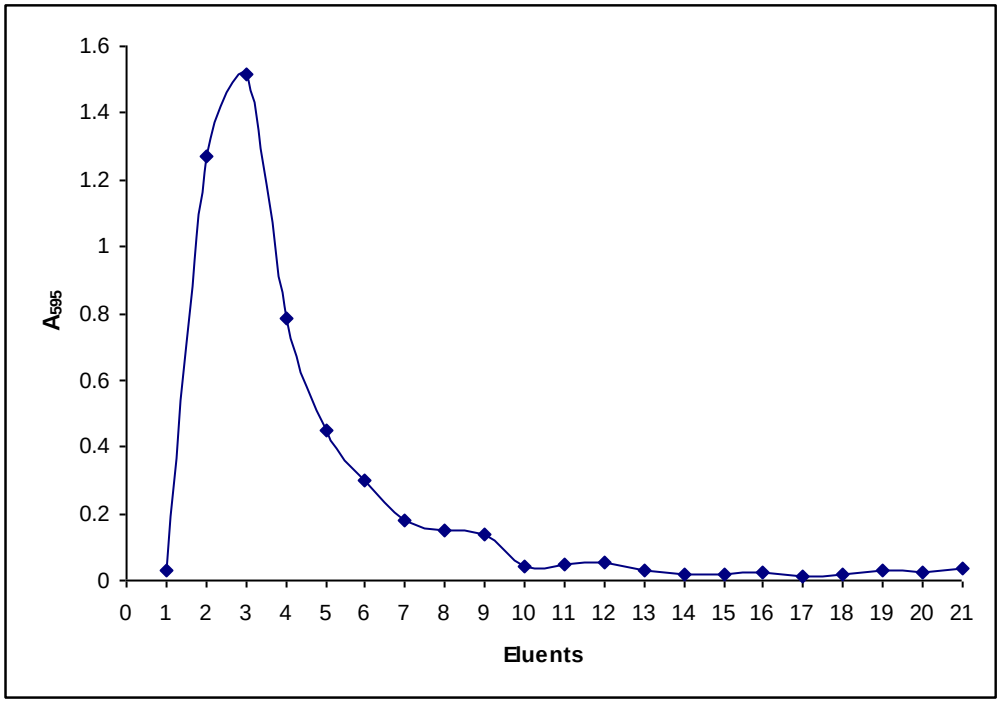
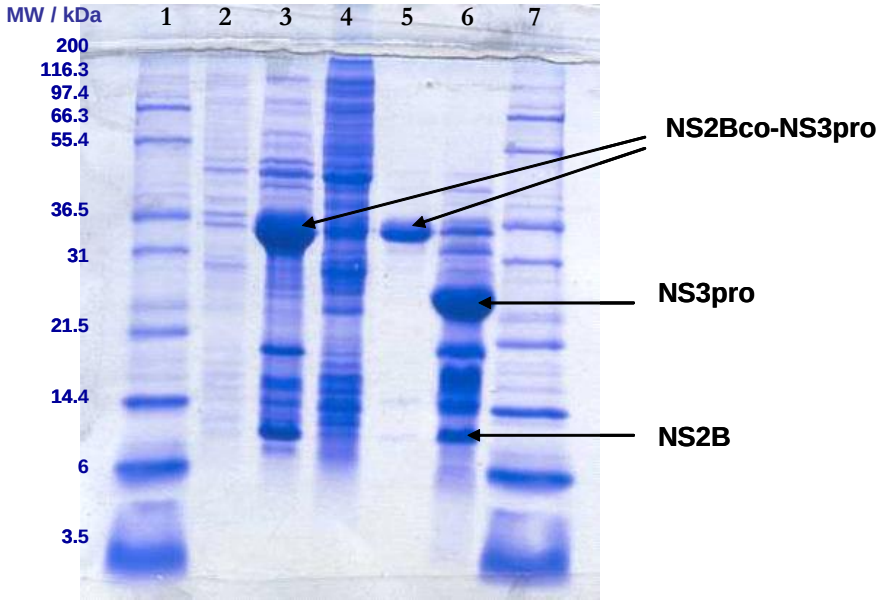
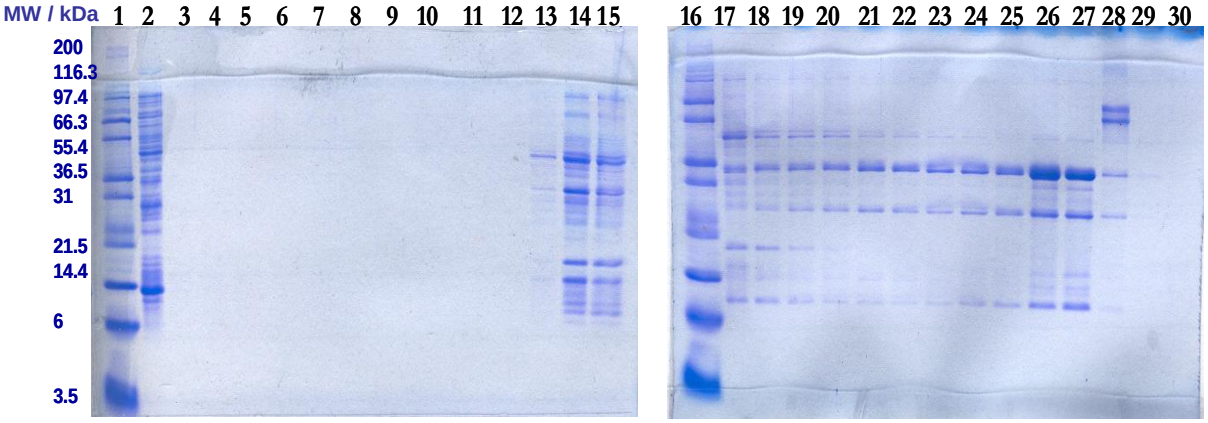


Figure 3.3 Elution profile of fractions obtained from purification of DEN-2 protease precursor on Nickel (Ni^{2+}) affinity column. Absorbance was measured at wavelength of 595 nm. Fractions 2 and 3 were further purified on gel filtration column.



Lane 1: Protein molecular weight marker
Lane 2: Soluble bacterial proteins after expression induced with IPTG
Lane 3: Fraction from Ni^{2+}-NTA affinity column eluted with 500 mM imidazole
Lane 4: Non-binding fraction on Ni^{2+}-NTA resin
Lane 5: Fraction from gel filtration column under denaturing condition using Hiload 16/60 Superdex 75 preparative grade
Lane 6: After dialysis, refolded protein precursor which underwent cleavage to generate NS2B (~ 8 kDa) and NS3pro (~ 23 kDa)
Lane 7: Protein molecular weight marker

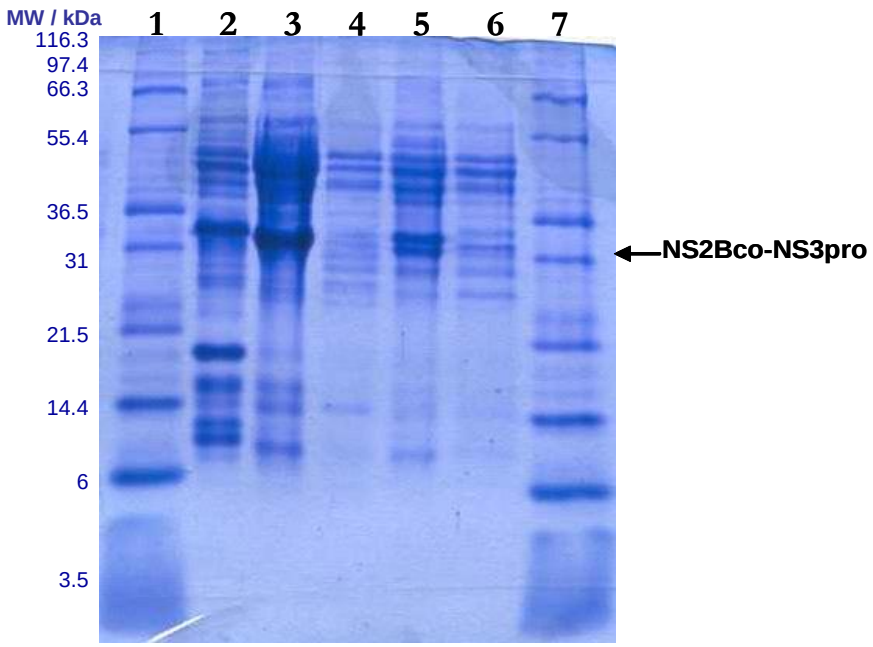
Figure 3.4 Expression and purification profile of NS2B-NS3pro illustrated by separation using 12 % SDS-PAGE and stained using coomassie blue.



Lane 1: Protein molecular weight marker
Lane 2: Non-binding fraction
Lane 3: Wash
Lane 4: 0.2 mM imidazole
Lane 5: 0.2 mM imidazole
Lane 6: 0.2 mM imidazole
Lane 7: 0.5 mM imidazole
Lane 8: 0.5 mM imidazole
Lane 9: 0.5 mM imidazole
Lane 10: 1 mM imidazole
Lane 11: 1 mM imidazole
Lane 12: 1 mM imidazole
Lane 13: 2 mM imidazole
Lane 14: 2 mM imidazole
Lane 15: 2 mM imidazole

Lane 16: Protein molecular weight marker
Lane 17: 3 mM imidazole
Lane 18: 3 mM imidazole
Lane 19: 3 mM imidazole
Lane 20: 4 mM imidazole
Lane 21: 4 mM imidazole
Lane 22: 4 mM imidazole
Lane 23: 5 mM imidazole
Lane 24: 5 mM imidazole
Lane 25: 5 mM imidazole
Lane 26: 20 mM imidazole
Lane 27: 20 mM imidazole
Lane 28: 20 mM imidazole
Lane 29: 50 mM imidazole
Lane 30: 50 mM imidazole

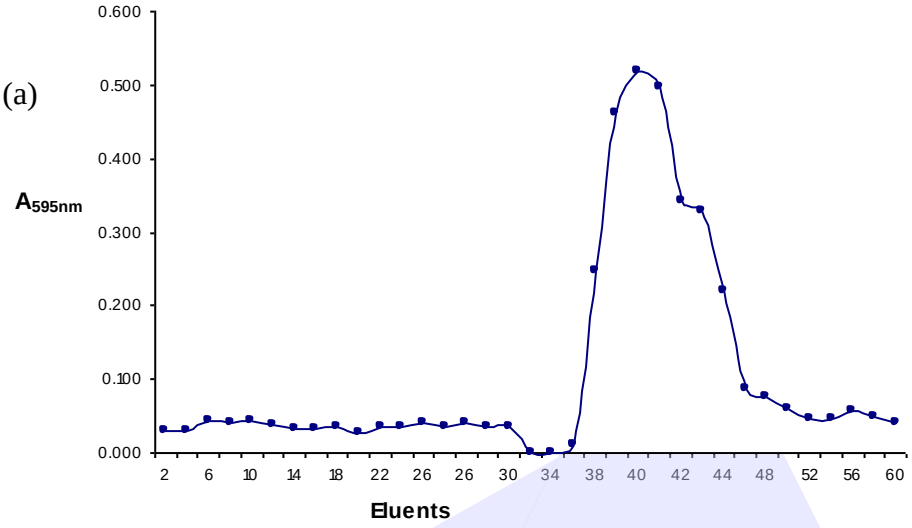
Figure 3.5 Profile of gradient elution of NS2B-NS3pro using different concentrations of imidazole as illustrated by 12 % SDS-PAGE.



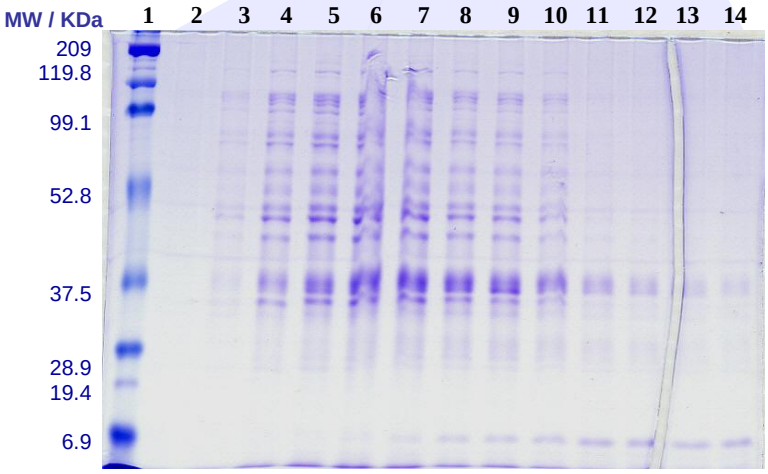
Lane 1: Protein molecular weight marker
Lane 2: Ni^{2+} affinity column (elution with 20 mM imidazole)
Lane 3: Gel filtration (denatured)
Lane 4: First dialysis (soluble fraction)
Lane 5: Second dialysis (pellet)
Lane 6: Second dialysis (soluble fraction)
Lane 7: Protein molecular weight marker

Figure 3.6 Profile of protein solution eluted from Ni^{2+} affinity column and dialysis products illustrated by 12 % SDS-PAGE.

(a)



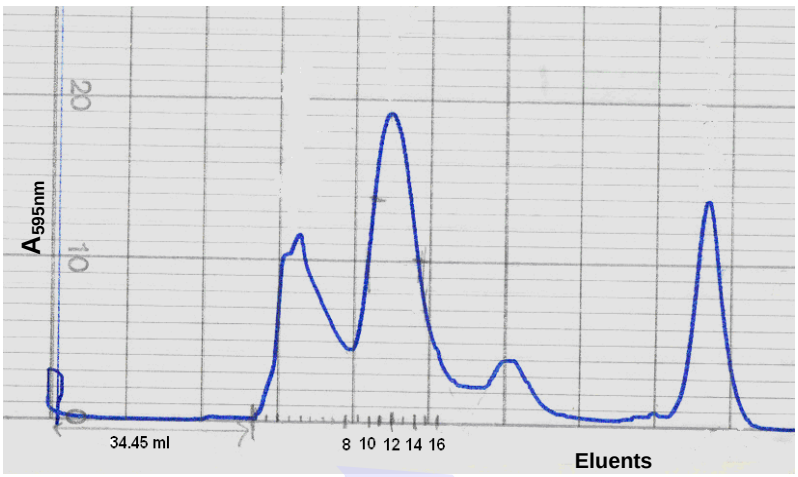
(b)



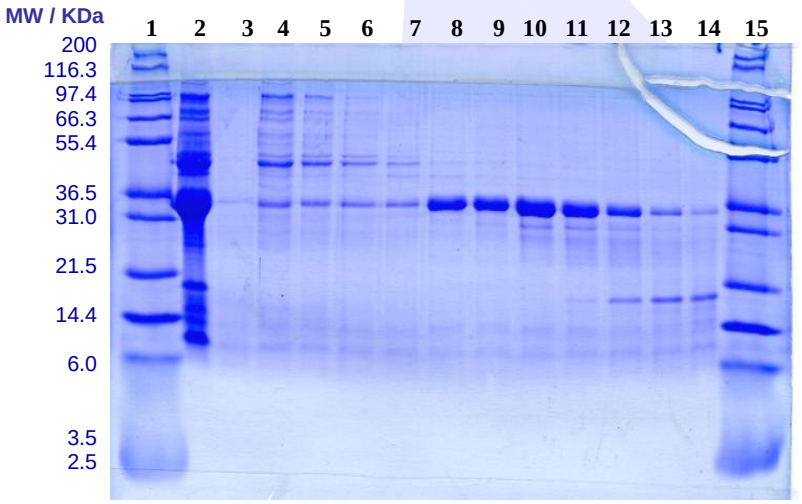
Lane 1: Protein molecular weight marker
Lane 2: Fraction 36
Lane 3: Fraction 37
Lane 4: Fraction 38
Lane 5: Fraction 39
Lane 6: Fraction 40
Lane 7: Fraction 41
Lane 8: Fraction 42
Lane 9: Fraction 43
Lane 10: Fraction 44
Lane 11: Fraction 45
Lane 12: Fraction 46
Lane 13: Fraction 47
Lane 14: Fraction 48

Figure 3.7 Elution profiles of eluents from size exclusion chromatography column using preparative Sephadex^{TM} G-75 (under denaturing condition and after subjected to Ni^{2+} affinity column). (a) Plot of protein concentration of the purified fractions (absorbance measured at 595 nm), and (b) 12 % SDS-PAGE showing fractions containing NS2Bco-NS3pro.

(a)



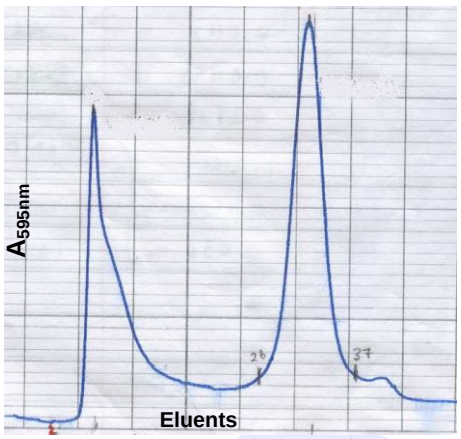
(b)



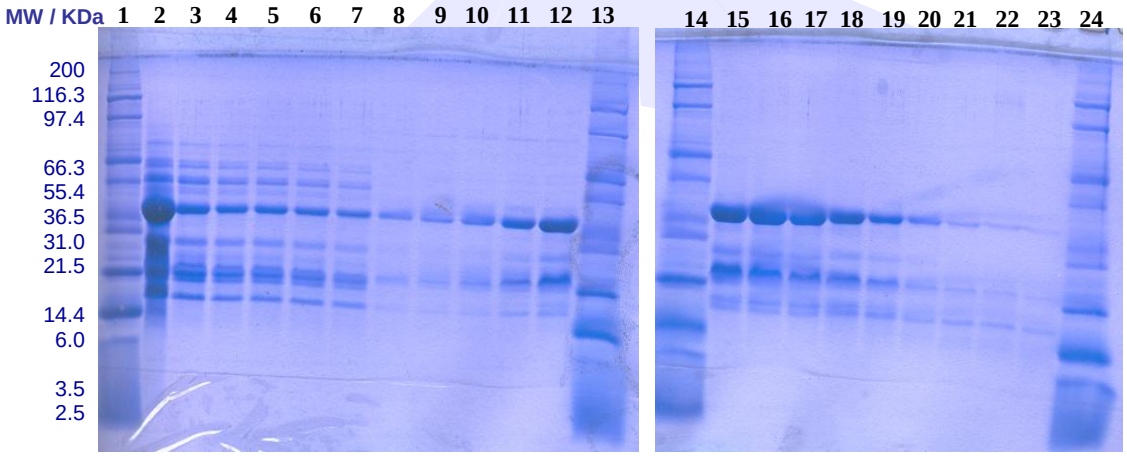
Lane 1:	Protein molecular weight marker	Lane 8:	Fraction 11
Lane 2:	Loading sample (dialysed)	Lane 9:	Fraction 12
Lane 3:	Fraction 1	Lane 10:	Fraction 13
Lane 4:	Fraction 3	Lane 11:	Fraction 14
Lane 5:	Fraction 5	Lane 12:	Fraction 15
Lane 6:	Fraction 7	Lane 13:	Fraction 16
Lane 7:	Fraction 9	Lane 14:	Fraction 17
		Lane 15:	Protein molecular weight marker

Figure 3.8 Elution profiles of eluents from size exclusion chromatography column using pre-packed Hiload Superdex^{TM} 200 column (under denaturing condition and after subjected to Ni^{2+} affinity column). (a) Plot of protein concentration of the purified fractions (absorbance measured at 595 nm), and (b) 12 % SDS-PAGE showing fractions containing NS2Bco-NS3pro.

(a)



(b)



Lane 1: Protein molecular weight marker
Lane 2: Loading sample (dialysed)
Lane 3: Fraction 6
Lane 4: Fraction 7
Lane 5: Fraction 8
Lane 6: Fraction 9
Lane 7: Fraction 10
Lane 8: Fraction 26
Lane 9: Fraction 27
Lane 10: Fraction 28
Lane 11: Fraction 29
Lane 12: Fraction 30
Lane 13: Protein molecular weight marker
Lane 14: Protein molecular weight marker
Lane 15: Fraction 31
Lane 16: Fraction 32
Lane 17: Fraction 33
Lane 18: Fraction 34
Lane 19: Fraction 35
Lane 20: Fraction 36
Lane 21: Fraction 37
Lane 22: Fraction 38
Lane 23: Fraction 39
Lane 24: Protein molecular weight marker

Figure 3.9 Elution profiles of eluents from size exclusion chromatography column using pre-packed Hiload Superdex^{TM} 200 column (under native condition). (a) Plot of protein concentration of the purified fractions (absorbance measured at 595 nm), and (b) 12 % SDS-PAGE of the purified fractions.

protease and its degenerative products. The peak on the left contained a less pure fraction, even though the presence of NS2B and NS3pro is observed, as shown by the SDS-PAGE in Figure 3.9b. Concentration of each fraction 29 to 35 was determined using Bio-Rad protein assay. Figure 3.10 shows the Bovine serum albumin standard curve used in the determination of protein concentrations in the fractions. Table 3.1 shows the protein yield after each purification step and the final overall protein yield.

3.3.2 Kinetic properties of DEN-2 NS2B-NS3pro

Biochemical properties, such as the type of buffers, optimum pH, and ionic strength greatly affect enzyme activities. The biochemical properties for performing *in vitro* assays of DEN-2 NS2B-NS3pro have been determined in previous studies (Hadinur, 2005; Teoh, 2004; Yusof *et al*., 2000). In this study, kinetic properties of the protease were determined to ensure that the protease, which was subsequently put through crystallization trials, was active. Based on the report by Yusof *et al.* (2000), the fluorogenic substrate, BOC-Gly-Arg-Arg-MCA, was used in this study. The cleavage of this substrate by the protease follows the Michaelis-Menten kinetics, and is dependent upon the incubation time and substrate concentration.

The amount of AMC released into the assay solution (in terms of intensity) upon cleavage of the substrate by the protease was determined from an AMC standard curve (Figure 3.11). A Lineweaver-burk plot, 1/V (the reciprocal of velocity), versus 1/[S], the reciprocal of substrate concentration, was plotted and found to be linear (Figure 3.12). Table 3.2 illustrates the different kinetic properties of DEN-2 NS2B-NS3pro obtained from this study. Each of the fractions was then concentrated down to about 10 mg/ml for crystallization screen.

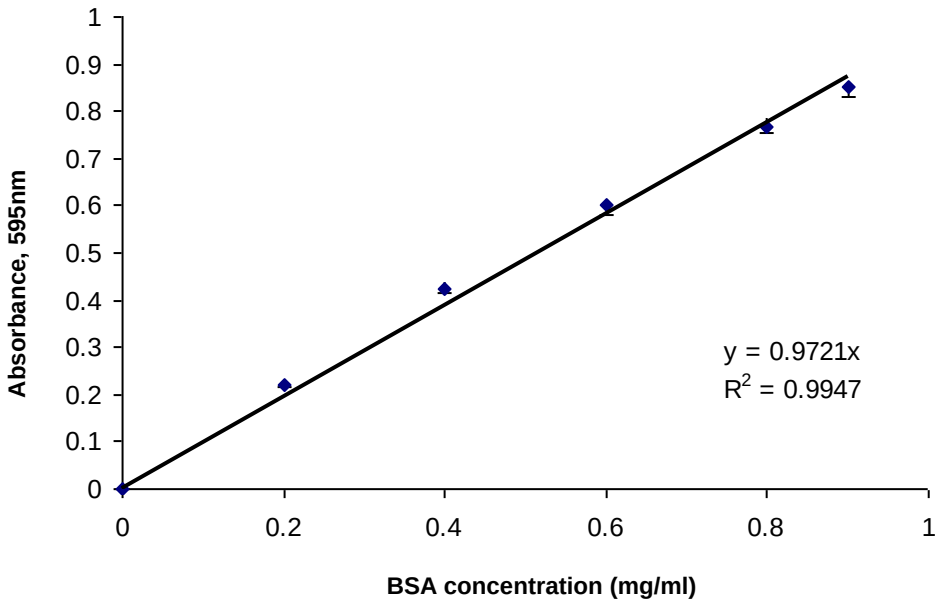


Figure 3.10 The Bovine serum albumin (BSA) standard curve. The linear range of the assay for BSA is 0.2 to 0.9 mg/ml. This plot was prepared and used to calculate the DEN-2 NS2B-NS3 protease complex concentration.

Table 3.1 Table showing protein yield after each step of purification and the overall protein yield.

	Technique acquired	Substance	Amount (weight)	Yield
1.	Overexpression & harvesting	Pellet	5000 mg	
2.	Extraction (lysis)	Total protein	397.97 mg	4.80%
3.	Immobilized metal (Ni^{2+}) affinity column	Purified protein	19.12 mg	
4.	Gel filtration chromatography (denatured)	Protein loaded Purified protein	16.75 mg (2 ml) 8.09 mg	48.30%
5.	Dialysis	Protein	5.48 mg	67.73%
6.	Gel filtration chromatography (native)	Protein loaded Purified protein	4.83 mg (400 µl) 2.28 mg	47.20%
	Overall yield			**0.74%**

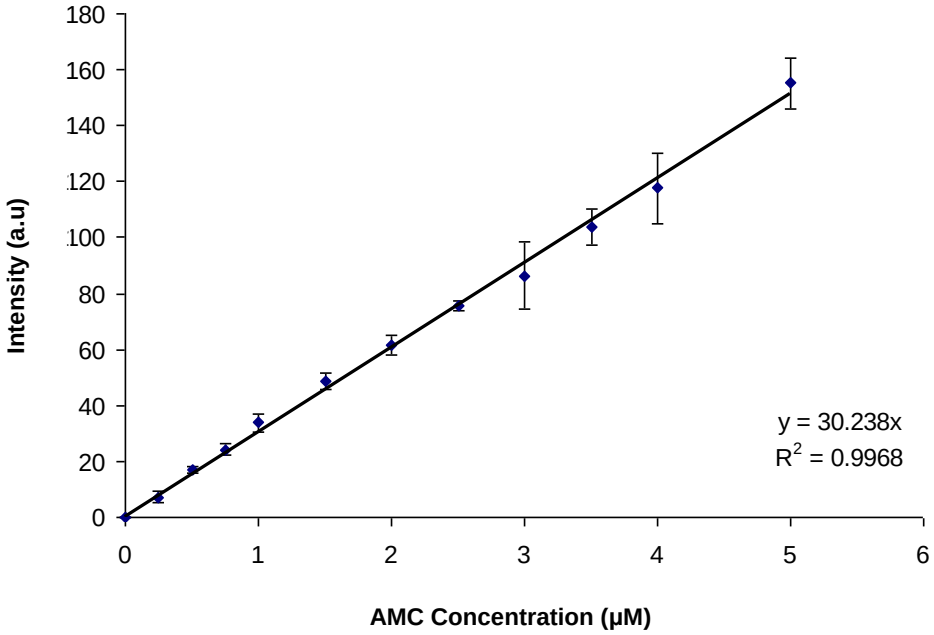


Figure 3.11 The 7-amino-4-methylcoumarin (AMC) standard curve. The AMC concentrations assayed range from 0.0 to 5.0 μM. Data are reported as mean ± standard deviation. This plot directly indicates the amount of AMC released into the assay solution, and hence, was used to characterize the NS2B-NS3pro kinetic properties. The standard curve assay was done in triplicate.

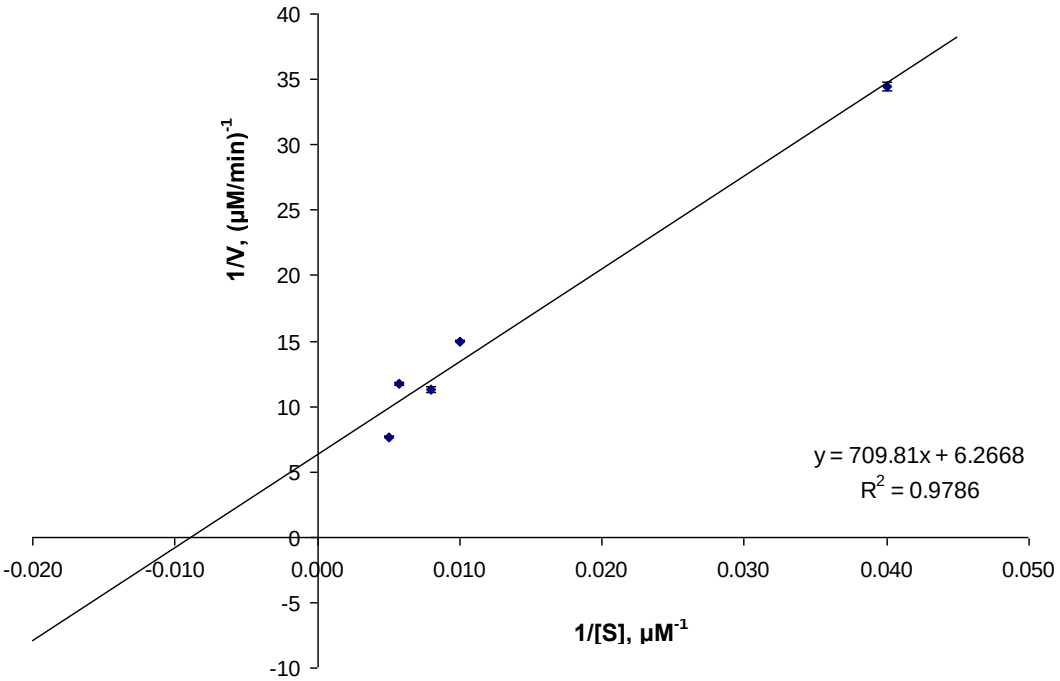


Figure 3.12 The Lineweaver-Burk plot for DEN-2 NS2B-NS3pro. The protease complex kinetic properties were determined from assays which consisted of the fluorogenic substrate (concentration ranging from 25 to 200 μM), protease concentration at 1 μM and buffer. Data are reported as mean ± standard deviation.

Table 3.2. Kinetic properties of NS2B-NS3pro.

V_{max}	K_m	k_{cat}	k_{cat}/K_m
0.16 μM min^{-1}	113.21 μM	0.0027 sec^{-1}	23.85 $M^{-1}sec^{-1}$

The kinetic properties were charaterized using the fluorogenic substrate, BOC-Gly-Arg-Arg-MCA, as described earlier (section 3.2.2.8). The maximum velocity, V_{max}, is the maximum number of reactions per unit time catalysed by one mole of protease (Garrett and Grisham, 1997a). The Michaelis constant, K_m, is defined as the substrate concentration at which velocity equals half the maximal velocity. The turnover number of the protease, k_{cat}, is a measure of the protease's catalytic activity. k_{cat} is defined as the number of substrate molecules converted to the product per protease molecule per unit time when the protease is saturated with substrate. k_{cat}/K_m is a measure of the catalytic efficiency of the protease operating at substrate concentrations substantially below saturation amounts. (See Appendix 2 for calculation methods).

3.3.3 Crystallization screens of DEN-2 NS2B-NS3pro

A total of 818 crystallization buffers were used in the crystallization screens of the DEN-2 NS2B-NS3pro. Crystallization attempts did not result in successful crystal formation. Upon incubation at 17^{o}C with the precipitating buffers, the protein solutions either formed clear solutions, precipitates or phase separation (Figure 3.13).

3.4 Discussion

In this chapter, we report the overexpression of DEN-2 protease complex in *Escherichia coli*, its purification and kinetic properties determination. Methods employed followed those reported by Yusof *et al*. (2000), with some optimization to ensure the protein yielded is of high purity and suitable for crystallization trials. The yield of the purified protease complex was considerably low at 0.74 % (Table 3.1). It was found, at the later stage of the study, that the low yield was due to the bacterial clone hosting the protein precursor losing its viability as a result of technical difficulties in the storage of the glycerol stocks.

The kinetic properties of the enzyme (Table 3.2) were found to be relatively poor compared to those reported by Yusof *et al*. (2000) where the values reported were 0.031 s^{-1} (k_{cat}), 180 μM (K_m) and 172 s^{-1} M^{-1} (k_{cat}/K_m). On the other hand, one dimensional profile of the purified protease via SDS-PAGE (Figure 3.4) illustrated the enzyme complex to be highly active upon purification which, after dialysis, refolded into its active conformation and underwent auto-cleavage to generate individual NS2B and NS3pro domains. This would signify that the recognition site (dibasic amino acids on the NS2B/NS3 junction) for binding to the active site of the protease was able to

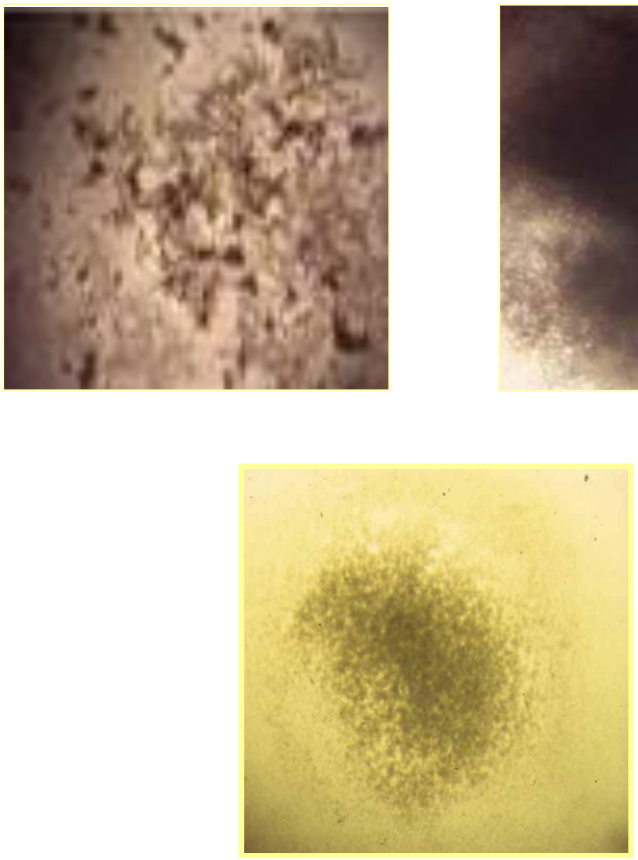


Figure 3.13 Pictures showing examples of the different forms of precipitates developed in the crystallisation screens of DEN-2 NS2B-NS3pro.

form interactions with the binding site for the cleaving process to proceed. This observation is substantial enough to allow the purified protease to be put through crystallization trials.

To check for possibility of mutation occurring, the plasmid carrying the DNA, which coded for the protease complex, was sent for sequencing. Figure 3.14a shows the translated amino acid sequences of NS2B-NS3pro from the DNA sequencing experiment. N-terminal amino acid sequencing was also performed on the purified protease complex (Figure 3.14b). The amino acid sequences shown in Figure 3.14 were exactly the same as those observed for the protease complex reported by Yusof *et al.* (2000). From Figure 3.14b, it can be seen that the active protease cleaved the NS2B/NS3 junction. In addition, the active protease is seen to cleave within the NS3 domain, where it recognized the basic arginine residue (R is underscored in Figure 3.14b), resulting in a band on the SDS-PAGE corresponding to protein with molecular weight 17 kDa. This finding is in agreement to that reported by Krishna Murthy *et al.* (1999) where they observed that from solvent accessibility calculations, NS3pro appeared to be in a partially "open" conformation. Here, the catalytic triad & residues in the substrate binding pocket were accessible to model substrates, thus allowing some level of intrinsic activity.

Crystallization experiments performed in this study, however, did not successfully lead to protein crystal formations. There was an instance where crystals were obtained (data not shown) using PEG/Ion Screen^{TM} #1 (refer Appendix 1). These crystals were observed as orthorhombic prisms and hexagonal plates. However, the diffraction patterns of the electron clouds obtained after the crystals were mounted on the single beam x-ray diffractometer (Rigaku Ru 2000 rotating anode, Rigaku/MSC,

(a)

3'5' Frame 3

XXXXXXGXPXNXIXXXPXXXXWGKXXEXXXGKXXGXXXCPXXXWXFXFPRXXGGQXGXQ
XXQKGNXXXPEFETXTXXFFQXXXXFXGVXXXXXKTXXXDFLXXKQXRGFRXTFXXKXPXD
X–KTXFYXXLIXLNIGVSRGPFXFXXRNHKNLFPLXADNNFTQNSLKRRXLTMRGSHHHHH
HGSSADLELERAADVKWEDQAEISGSSPILSITISEDGSMSIKNEEEEQTLWYLWEVKKQR
AGVLWDVPSPPPVGKAELEDGAYRIKQKGILGYSQIGAGVYKEGTFHTMWHVTRGAVLM
HKGKRIEPSWADVKKDLISYGGGWKLEGEWKEGEEVQVLALEPGKNPRAVQTKPGLFKT
NAGTIGAVSLDFSPGTSGSPIIDKKGKVVGLYGNGVVTRSGAYVSAIAQTEKSIEDNPEIED
DIFRKKLN–LSLDSC– –IQ– –QNSXGFFRTLGCAG

(b)

NS3(pro)
AGVLWDVPSPPPVGKAELEDGAYRIKQKGILGYSQIGAGVYKEGTFHTMWHVTRGAVLMHKGKRIEP
SWADVKKDLISYGGGWKLEGEWKEGEEVQVLALEPGKNPRAVQTKPGLFKTNAGTIGAVSLDFSPG
TSGSPIIDKKGKVVGLYGNGVVTRSGAYVSAIAQTEKSIEDNPEIEDDIFRK (20 kDa)

AGVLWDVPSPPPVGKAELEDGAYRIKQKGILGYSQIGAGVYKEGTFHTMWHVTRGAVLMHKGKRIEP
SWADVKKDLISYGGGWKLEGEWKEGEEVQVLALEPGKNPRAVQTKPGLFKTNAGTIGAVSLDFSPG
TSGSPIIDKKGKVVGLYGNGVVTR (17 kDa)

6H-NS2B
GSHHHHHHGSSADLELERAADVKWEDQAEISGSSPILSITISEDGSMSIKNEEEEQTLWYLWEVKKQR
(8 kDa)

Figure 3.14 (a) Translated amino acid sequences of NS2B-NS3pro as a result of DNA sequencing experiment carried out on the plasmid carrying the DNA coding for the protease complex. The sequencing was done by Lark Technologies, Inc., Essex, UK. The sequences in blue colour represent the NS2B domain, while those in pink represent the NS3pro domain. It can be seen that there are six histidine residues on the N-terminus of NS2B which serve as a tagging to facilitate the protein purification using immobilized metal-affinity chromatography (refer section 3.2.2.3). (b) Results from N-terminal protein sequencing of the purified protein after dialysis (refolded into its active conformation). Sequencing was done at the Department of Molecular Biology and Biotechnology, the University of Sheffield. The different sets of sequences were matched to the SDS-PAGE profile of the purified protease according to the weight, as shown in parenthesis.

TX, U.S.A., with MAR345 Image Plate Detector) confirmed the crystals to be the salts of the precipitating buffer, and not of the purified protease.

Ironically, after all our attempts to crystallize the protease complex went futile and a homology model of the protease (not reported in this thesis) was generated for the next part of the study, a crystal structure data for the DEN-2 NS2B-NS3 protease complex was deposited into the protein data bank (PDB) (D'Arcy *et al.*, 2006; Erbel *et al.*, 2006). D'Arcy and co-workers genetically engineered the protease expression construct to make a 47-amino-acid hydrophilic core sequence assembly of NS2B (amino acids 1394–1440) linked via a nine-amino-acid linker (G4-S-G4) to the N-terminal 185 amino acids of NS3 (amino acids 1476–1660). This construct led to the overexpression of a highly soluble protease complex which was then concentrated to the very high concentration of 60 to 80 mg/ml for crystallization trials. This expressed protease also did not undergo autocleavage upon folding to its active conformation due to the removal of the dibasic amino acids upstream of the NS2B/NS3 junction (by replacing the 10 amino acids upstream the N-terminal of NS3 with the linker).

In our study, one of the problems encountered was due to a highly dynamic protease that underwent autocleavage as soon as it resumed its active conformation. This led to a non-homogenous solution, a highly unfavourable condition for protein crystal formation. In addition, the protease complex was not so soluble in buffer. Concentration beyond 10 mg/ml resulted in precipitation. Replacement of the 10 amino acids upstream the N-terminal of NS3 (Figure 3.14; these amino acids are coded in dark blue) with the linker, as reported by the Novartis team, had resolved the solubility problem, thus their success in the crystallization trials.

3.5 Conclusion

In this study, the methods for purification of DEN-2 NS2B-NS3 protease in an attempt to obtain pure protein solutions suitable for crystallization screens were optimized. However, crystallization trials did not result in any protein crystal formation. The reasons for this were, firstly, the protease was not highly soluble in buffer and, secondly, the protease was highly dynamic and underwent autocleavage at NS2B/NS3 junction as soon as it folded into its active conformation. Hence, crystallization trials were not successful due to purity problem (high degree of purity is required to obtain any initial hits) and homogeneity problem of the protein solution, where other degenerative products were present in the solution due to the cleaving process.

Subsequently, for projects on molecular modelling in this study of DEN-2 protease, the model built from crystal data deposited into the PDB (PDBid: 2FOM) by Erbel *et al.* (2006) was used as the target.