Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2021.082

RESEARCH ARTICLE

Enhanced Solubilization and Purification of 3ABC Non-structural Protein of Foot-and-Mouth Disease Virus from Bacterial Inclusion Bodies

Muhammad Ashir Zia[1, 2*], Muhammad Salahuddin Shah[1, 2] and Mudasser Habib[1,2*]

[1]College of Biological Sciences, Nuclear Institute for Agriculture and Biology College, (NIAB-C), Pakistan Institute of Engineering and Applied Sciences, Nilore, 44000, Islamabad; [2]Vaccine Development Group, Animal Sciences Division, Nuclear Institute for Agriculture and Biology, Jhang road, P.O box 128, Faisalabad, 38000 Pakistan
*Corresponding author: ashirzia68@gmail.com; mudasserhabib@yahoo.com

ARTICLE HISTORY (21-319)

Received: July 17, 2021
Revised: November 17, 2021
Accepted: December 03, 2021
Published online: December 20, 2021

Key words:
3ABC
FMDV
Inclusion bodies
non-ionic detergent
NSP
Stability

ABSTRACT

Nonstructural 3ABC protein of foot-and-mouth disease virus (FMDV) is used to differentiate vaccinated from naturally infected animals. The 3ABC protein is a polyprotein which is cleaved into membrane associated 3A protein, three copies of 3B and 3Cpro mediated by virally encoded 3C protease. The expression of this protein in *E. coli* results into the formation of inclusion bodies which require solubilization in high concentration of chaotropes and extensive refolding process prior to purification of the native protein. Protein aggregation during refolding leads to the poor recovery of protein in functional form. Alternatively, mild solubilization methods have been proposed to recover the native and soluble protein from inclusion bodies present in *E. coli*. In this study, 3ABC protein was expressed predominantly as inclusion bodies using *E. coli* host and solubilized in mild non-ionic detergent followed by purification through Ni-NTA chromatography. The protein recovery using this solubilization method, showed higher yield than previous solubilization methods for 3ABC protein. This method also favored higher stability of the 3ABC recombinant protein stored at different temperatures. The reactivity of the proteins was analyzed by western blotting and ELISA which showed their ability to use them as antigen for the development of immunoassays. In conclusion, this study demonstrates an efficient and high yielding purification method of this protein without refolding process than previously described methods involving renaturation steps.

To Cite This Article: Zia MA, Shah MS and Habib M, 2022. Enhanced solubilization and purification of 3ABC non-structural protein of foot-and-mouth disease virus from bacterial inclusion bodies. Pak Vet J, 42(1): 74-80. http://dx.doi.org/10.29261/pakvetj/2021.082

INTRODUCTION

Foot-and-mouth disease virus (FMDV) belongs to *Aphthovirus* genus and *Picornaviridae* family that causes a highly contagious vesicular disease in cloven-hoofed animals. The genome of this virus is single stranded, positive sense RNA with about 8.5 kb in length and encodes for a large single polyprotein. The polyprotein is cleaved into four capsid forming structural proteins (1A, 1B, 1C, and 1D) and eleven non-structural proteins (L(L_b and L_{ab}), 2A, 2B, 2C, 3A, 3B (1-3), 3C and 3D) that play important role in virus replication (Mason *et al.*, 2003, Gao *et al.*, 2016). The control of this disease mainly relies on routine vaccination with inactivated whole virus vaccines in the endemic countries. These vaccines mainly contain structural proteins (SPs) and are devoid of non-structural proteins (NSPs). The antibodies are directed to structural proteins only following vaccination hence, the detection of NSPs in reliable indicator of exposure with live replicating virus (Tewari *et al.*, 2021). Among different NSPs, the detection of non-structural 3ABC polyprotein is considered as reliable in the differentiating the infected animals in vaccinated population. This protein is highly immunogenic and induces long term antibodies (Lu *et al.*, 2007; Gelkop *et al.*, 2018).

FMDV NSP 3ABC is a polyprotein which is cleaved into membrane associated 3A protein, three copies of 3B and 3Cpro mediated by virally encoded 3C protease. This protein is expressed as recombinant protein using either *E. coli* (Srisombundit *et al.*, 2013) or Baculovirus (Hosamani *et al.*, 2015) expression hosts to develop different formats of ELISA tests. The expression of 3ABC protein in *E. coli* leads to the formation of inclusion bodies due to its poor solubility and high hydrophobicity in the polyprotein

structure (Salem *et al.*, 2021). Inclusion bodies are insoluble aggregates of misfolded polypeptide in *E. coli* following over-expression of protein. Some proteins expressed in inclusion bodies might contain native-like structures which can be solubilized and renatured by refolding to give the native structure (Kaur *et al.*, 2018) . Normally, Urea and guanidine hydrochloride are used for solubilizing the protein aggregates followed by refolding either by dilution or refolding in the packed columns (Singh and Panda, 2005). However, refolding is not routinely used for membrane protein. Membrane proteins mainly with α-helices which are highly hydrophobic in nature and resistant to the chemical denaturation, are difficult to solubilize the proteins without hindering the denaturant removal and protein refolding (Barrera *et al.*, 2005). High concentration of chaotropes like urea and guanidine hydrochloride (GdnHCl) completely denatures the existing secondary structures which often leads to the protein aggregation during refolding (Rudolph and Lilie, 1996). Recovery and refolding of protein from inclusion bodies using mild solubilization strategies are effective to solubilize the proteins than reported alternatively in comparison with high concentration of chaotropes. The mild solubilization technique retains the existing secondary structures of proteins (Vinogradov *et al.*, 2003). Many proteins such as aquaporin which are integral membrane associated proteins, have been produced in milligrams using mild detergent solubilization (Wang *et al.*, 2015). Another strategy for purifying proteins from IBs involves their overexpression using highly soluble proteins as fusion partners, such as glutathione-S-transferase (GST), Maltose binding protein (MBP), and Thioredoxin. However, protein instability of the target protein is major problem during cleavage of fusion tag (Francis *et al.*, 2012). Other hosts such as baculovirus favors the soluble expression of heterologous proteins but unlike *E. coli*, they are expensive, requires cell culture and often produce low yield of protein (Tripathi and Shrivastava, 2019; Zeedan *et al*., 2020).

Previously, inclusion bodies containing 3ABC protein were solubilized using high concentration of urea followed by refolding steps to remove excess urea from the protein (Gelkop *et al.*, 2018). The use of high amount of refolding buffer and low refolding yields most likely due to protein aggregation are the limitations to use this solubilization procedure (Tsumoto *et al.*, 2003). During refolding, low protein concentration and reduced intermolecular interactions are essential to inhibit the aggregation of proteins and increase the refolding yield (Schramm *et al.*, 2020). Moreover, this protein was solubilized in low concentration of ionic detergent such as N-lauryl sarcosine (NLS) which was essential for solubilizing the inclusion bodies. The 3ABC protein solubilized in NLS buffer was purified without using refolding steps (Lu *et al.*, 2007). It is also reported that purified protein in the buffers containing NLS may be in inactive form which require refolding prior to functional analysis (Song *et al.*, 2009). Moreover, higher concentrations of NLS (>0.3%) may inhibit the binding to Ni^{2+} failing to purify through Ni-NTA chromatography. Therefore, NLS can be replaced by non-ionic detergent in order to improve the purification yield (Francis *et al.*, 2012). Previously, the effect of non-ionic detergent has not been studied for the solubilization of 3ABC protein expressed as inclusion bodies in *E. coli*.

In this study, we have employed mild solubilization strategy with non-ionic detergent, n-Dodecyl-B-D-Maltoside (DDM) and compared with previous solubilization methods for recombinant 3ABC protein. The solubilized proteins are purified through Ni-NTA chromatography and compared their purification yields. These proteins are characterized by immunoblotting and ELISA using FMD specific sera confirmed by commercial ELISA kit. To check protein stability, the protein activity is compared at different temperatures to analyze any loss in the reactivity.

MATERIALS AND METHODS

Expression and solubility analysis of recombinant 3ABC protein in *E. coli:* Recombinant 3ABC construct was generated from FMDV type O isolate (Accession number: KY192528) using pET-28a-SUMO (Novagen) expression vector. The orientation of the insert in the resultant construct was confirmed by Sanger DNA sequencing which was performed by First BASE Laboratory Sdn Bhd (Selangor, Malaysia). Following transformation into BL21 DE3 cells, a single transformed colony was grown overnight at 37ºC in LB media containing 50 µg/mL kanamycin. The overnight culture was diluted 1:100 into fresh LB media with 50 ug/mL kanamycin and incubated with shaking until O.D. reached to 0.6. The culture was induced with 1mM IPTG and incubated at 37ºC for four hours. Induced culture was centrifuged at 4000 rpm for 15 minutes at 4ºC using VersatiTM refrigerated centrifuge (ESCO, Singapore).

To check solubility, induced culture pellets was resuspended in lysis buffer (50mM Tris pH 8.0, 300 mM NaCl, 5mM imidazole) and cells were lysed by sonication (Qsonicator500, Qsonica, USA). Lysate was centrifuged at 12000 rpm for 20 minutes at 4ºC to separate supernatant (soluble protein) and pellet (insoluble protein). Test expression and solubility of expressed protein was checked on SDS-PAGE.

Solubilization of insoluble protein in different detergents: Insoluble protein fraction was solubilized in 20 mL/litre culture of solubilization buffer (50mM Tris pH 8.0, 300 mM NaCl, 5mM imidazole, 5% Glycerol) supplemented with 0.1% DDM. Cells were lysed by sonication and centrifuged at 12000 rpm for 40 minutes at 4ºC. Insoluble 3ABC protein aggregates were also solubilized using 0.3% NLS (Lu *et al.*, 2007) and 8M urea (Foord *et al.*, 2007) as described previously. Solubilized 3ABC proteins were used for purification.

Purification of recombinant 3ABC fusion protein: Purified protein fraction solubilized in 0.1% DDM was incubated with 1mL Ni-NTA agarose beads (Qiagen, Germany) for one hour with gentle shaking and loaded on column equilibrated with solubilization buffer. The column was washed once with wash buffer containing 25 mM imidazole. Elution of protein was performed using elution buffer containing different imidazole concentrations i.e. 200mM, 300mM and 500mM. Solubilization of protein in urea and NLS were also

purified using same protocol described above except replacing DDM with 8M urea and 1% NLS. Purity of protein was analyzed on SDS-PAGE and protein concentration was determined using Bradford microplate assay with bovine serum albumin as standard (Kruger, 2009). Briefly, BSA standards such as 2 mg/mL, 1.5 mg/mL, 1 mg/mL, 0.5 mg/mL, and 0.25 mg/mL were prepared using the respective elution buffer. One milliliter of Bradford's reagent (4.75% of 95% Ethanol, 8.5% of 85% Phosphoric acid and 1.2 mM coomassie brilliant blue G-250) was added into each well and 20 µL standards or protein samples were added into the respective wells with gentle mixing. After incubation for ten minutes at room temperature, the absorbance of each standard or sample was measured at 595nm. A standard curve was generated by plotting average absorbance at 595nm as a function of protein standards concentrations to determine the concentrations of unknown protein samples.

Immunoblotting: Purified protein fractions were separated by SDS-PAGE and transferred to the nitrocellulose membrane (BioTrace™ NT, Mexico) for two hours at constant 350 mA current and 22 volts using semi-dry method (Semi dry blotting units, Cleaver Scientific Ltd, UK). Membrane was blocked with 3% blocking solution (3% BSA in 1X TBST) at room temperature for one hour followed by washing three times with 1X TBST. Membrane was incubated with FMDV positive serum diluted 1:500 in blocking solution at room temperature for two hours. Following washing, membrane was incubated with Alkaline Phosphatase-conjugated rabbit anti-bovine IgG (Abnova™) diluted 1:3000 in blocking solution for one hour at room temperature. After washing, blot was developed by applying BCIP/NBT substrate until bands appeared and reaction was stopped with 5mM EDTA.

ELISA: Total 31 serum samples were collected from FMDV positive (n=15) and negative sera (n=16) which were confirmed using commercial kit (ID Screen® FMD NSP Competition, France). Positive and negative controls provided with the commercial kit, were also included to make sure that experiment worked. ELISA plate was coated overnight at 4ºC with 1 µg/ml of recombinant 3ABC proteins prepared in 50 mM carbonate/bicarbonate buffer, pH 9.6. Following washing three times with PBST (PBS with 0.05% Tween-20), plate was blocked with blocking buffer (2% BSA-V in PBST) and incubated at 37ºC for one hour. After four washes, serum samples diluted 1:20 in blocking buffer were added and incubated at 37ºC for one hour. Following five washes, plate was incubated at 37°C for one hour with HRP-conjugated rabbit anti-cow IgG diluted 1:3000 in blocking buffer. Finally, color was developed with TMB/H_2O_2 for 10 minutes reaction was stopped with stop solution (1M H_2SO_4). Absorbance at 450 nm was determined using a microplate reader (Ledetect 96, Austria). Results were expressed as O.D.450nm and calculated using following formula:

$$O.D\ 450\text{nm} = \frac{Abs\ Sample - Abs\ NC}{Abs\ PC - Abs\ NC}$$

Thermal Stability of recombinant protein at different temperatures: Thermal stability of purified recombinant 3ABC proteins was tested by storing at -20ºC, 4ºC and 37° C up to 60 days. On same ELISA plate, proteins stored at −70ºC and those stored at higher temperatures were tested against positive serum. PP values were calculated using O.D. values obtained from antigens stored at higher temperatures stored antigens in relation to the O.D. values obtained with −70°C stored antigens.

$$\text{PP value \%} = \frac{\text{O.D. proteins stored at (-20ºC, 4ºC or 37ºC)}}{\text{O.D. protein stored at -70ºC}} \times 100$$

RESULTS

Analysis of expression and solubility of recombinant 3ABC protein: Gene corresponding to 3ABC polyprotein was amplified by PCR, cloned into pET expression vector and transformed into BL21 DE3 cells. DNA sequencing revealed the correct orientation of 3ABC gene as in frame with fusion partners. The expression of recombinant protein was achieved using IPTG induction method and checked on SDS-PAGE. The expressed protein showed expected size of 70 kDa (55kDa of target protein and 15 kDa SUMO tag) on SDS-PAG. This band was absent in the control bacterial extract with no IPTG. Following sonication, it was observed that the recombinant 3ABC protein was expressed predominantly in insoluble fraction (as inclusion bodies) than soluble fractions (Fig 1). For soluble expression, the expression of protein was performed at different conditions i.e. temperatures, IPTG concentrations and post-induction time but the 3ABC protein was expressed mainly in inclusion bodies and exerted no effect on the solubility of protein (data not shown). Therefore, the expression of recombinant 3ABC protein was performed at 1mM IPTG at 37ºC for four hours.

Effect of detergents on solubility of recombinant 3ABC protein: Inclusion bodies containing insoluble 3ABC protein were solubilized in three different detergents i.e. urea, NLS and DDM. Urea and NLS were used controls for solubilizing the inclusion bodies since they were used previously for 3ABC protein, therefore their efficiencies were compared with another detergent, DDM. The insoluble 3ABC pellets were solubilized in 0.1% DDM, 8M urea, and 0.3% NLS. Inclusion bodies containing 3ABC protein were solubilized and centrifuged. The resultant supernatants were analyzed on SDS-PAGE to check solubilization efficiencies of three detergents. It was observed that maximum protein was solubilized in soluble form with DDM as compared with NLS and urea (Fig 2A).

Purification of recombinant 3ABC protein: The recombinant 3ABC protein was purified through Ni-NTA chromatography using these solubilized supernatants and the purity was analyzed on SDS-PAGE. SDS-PAGE analysis showed the estimated molecular weight of about 70 kDa indicating the purified 3ABC protein. Higher purification of recombinant 3ABC protein was obtained using DDM solubilized supernatant than NLS- and urea-solubilized supernatants. It was observed that higher

purification was achieved by using elution buffer with 200 mM imidazole than the buffers containing 300mM and 500 mM imidazole concentrations (Fig 2B). The purified proteins were quantified by Bradford assay (Fig 3) which showed that higher protein yield of 8 mg obtained from DDM solubilized supernatant followed by 5.2 mg and 3.25 mg from NLS- and urea-solubilized supernatants, respectively (Table 1). The specificity of recombinant 3ABC proteins were also confirmed by immunoblotting using FMDV specific serum obtained from infected animal. The western blot analysis showed the presence of 70 kDa protein band which indicated the good reactivity of anti-3ABC antibodies with FMDV positive serum (Fig 2C). This reactivity indicates that this protein can be used for the development of immunoassays for detecting the virus-specific antibodies.

Characterization of recombinant protein through ELISA: To check the diagnostic ability in DIVA analysis, recombinant 3ABC protein was characterized on ELISA using serum samples from infected and healthy animals. These serum samples were confirmed by commercial ELISA kit. It was observed that all the serum samples showed similar results as compared with commercial ELISA kit which indicated that these proteins were capable to differentiate between vaccinated/healthy and infected animals. The serum samples from FMDV infected animals showed strong reactivity with 3ABC polyprotein while no reactivity was observed in the sera from healthy animals. The highest to lowest O.D. values obtained from positive serum samples were ranging from 1.02 to 0.65, 1.08 to 0.73 and 0.99 to 0.65 when tested with NLS, DDM and urea containing proteins respectively. On the other hand, lowest to highest O.D. values from negative serum samples were ranging from 0.016 to 0.18, 0.02 to 0.19 and 0.02 to 0.19 after analyzing with NLS, DDM and urea containing proteins. The O.D. values of positive control was 0.91, 0.94 and 0.88 was obtained from purification with NLS-, DDM- and urea-solubilized supernatants respectively. While, negative control serum showed O.D. values of 0.12, 0.09 and 0.14 with purified proteins fractions from NLS-, DDM- and urea-solubilized supernatants respectively (Fig 4A and 4B).

Evaluation of thermal stability of recombinant 3ABC protein: Thermal stability of purified proteins was analyzed by storing proteins at different temperatures i.e. -20ºC, 4ºC and 37ºC for 60 days. The drop in the reactivity of tested proteins were considered as significant if 20 % reduction in the PP values were observed between proteins stored at different temperatures compared with those stored at -70ºC (Mahajan *et al.*, 2013). Both DDM and NLS-solubilized proteins showed no significant drop in their reactivity when stored at different temperatures. DDM-solubilized proteins showed maximum reduction in PP values of 84% at day 50, 81% at day 50 and 80% at day 30 when stored at -20ºC, 4ºC and 37ºC respectively (Fig 5A). NLS containing protein showed lowest PP value upto 79% at day 30, 75% at day 60 and 76 % at 60 after storing at -20ºC, 4ºC and 37ºC respectively (Fig 5B). The maximum drop in PP values of 78% at day 60, 72% at day 50 and 75% at 60 was observed in urea containing protein stored at -20ºC, 4ºC and 37ºC respectively (Fig 5C).

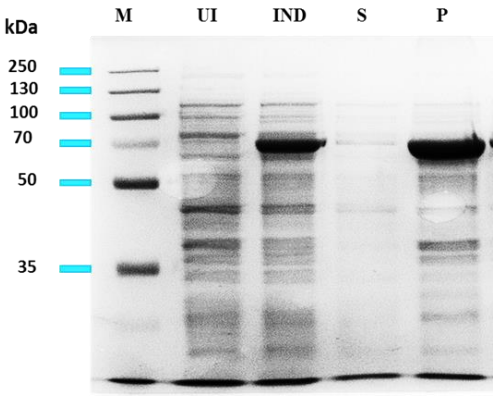

Fig 1: SDS-PAGE analysis of the expression and solubility of recombinant 3ABC protein in *E. coli*. Lane M, Protein Marker. UI, un-induced cell extract. IND, induced cell extract. S, soluble protein fraction after sonication. P, Insoluble protein fraction after sonication. M, Pre-stained Protein Marker.

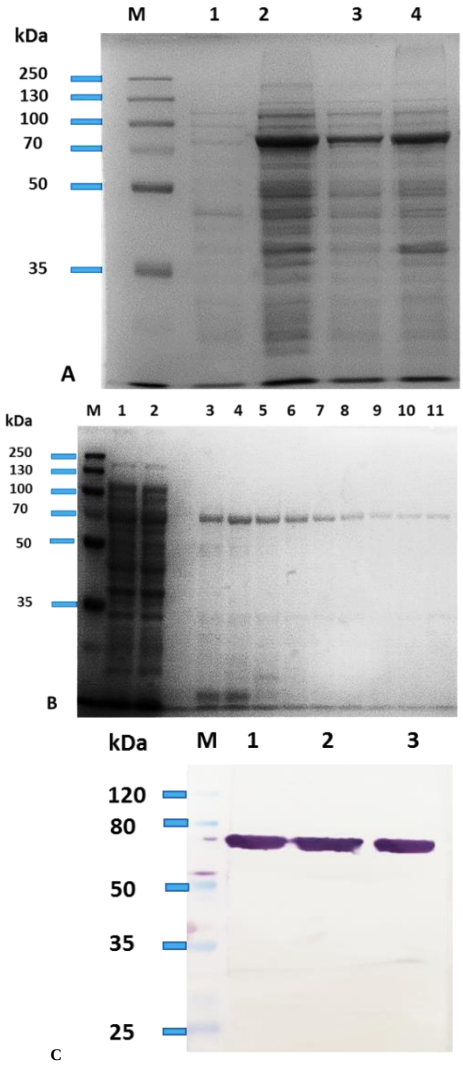

Fig 2: SDS-PAGE profile of solubility and purification of recombinant 3ABC fusion protein. A) Analysis of solubilizing insoluble pellet in DDM (lane 2), Urea (Lane 3) and NLS (Lane 4). Lane 1, insolubilized pellet. B) Purification of recombinant 3ABC protein through Ni-NTA chromatography. Lane 1 & 2. Solubilized supernatants containing DDM and NLS respectively. Lanes 3, 4, & 5. Purified protein fractions from DDM-solubilized supernatant. Lanes 6, 7, & 8. Purified protein fraction from NLS-solubilized supernatant. Lanes 9, 10, &11. Purified protein fraction from urea-solubilized supernatant. C) Western blot analysis of recombinant 3ABC protein. Lane 1, 2 & 3, Purified protein fractions from urea-, DDM- and NLS-solubilized supernatants.

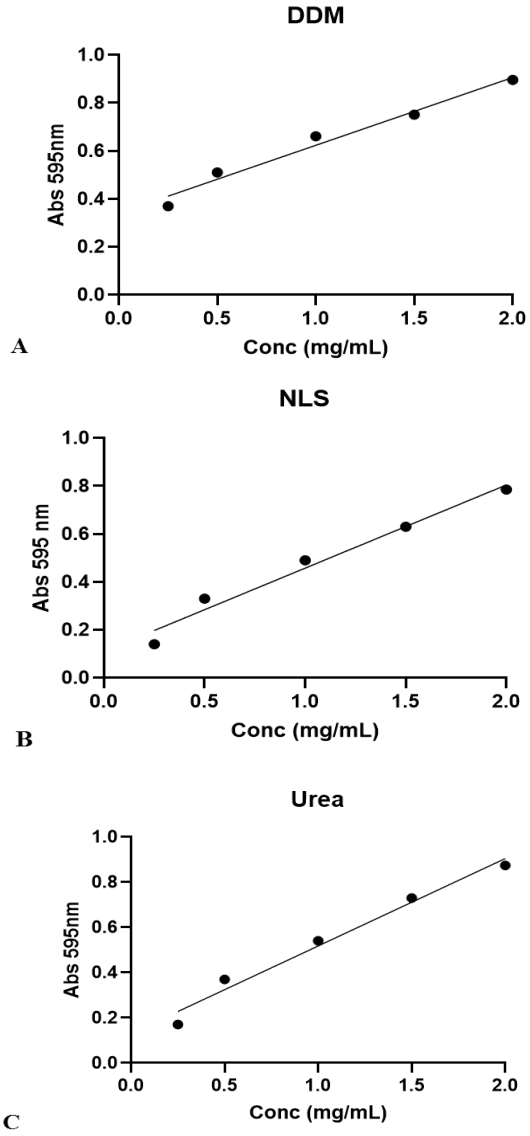

Fig. 3: Bradford standard curves using bovine serum albumin (BSA) as standards. The linear equations are Y=0.2827x+ 0.3407 with R^2=0.9750, Y=0.347x+0.1107 with R^2=0.9731, and y=0.3865x+0.131 with R^2=0.9771 for DDM (A), NLS (B) and Urea (C) respectively, to determine protein concentrations after purification from respective supernatants.

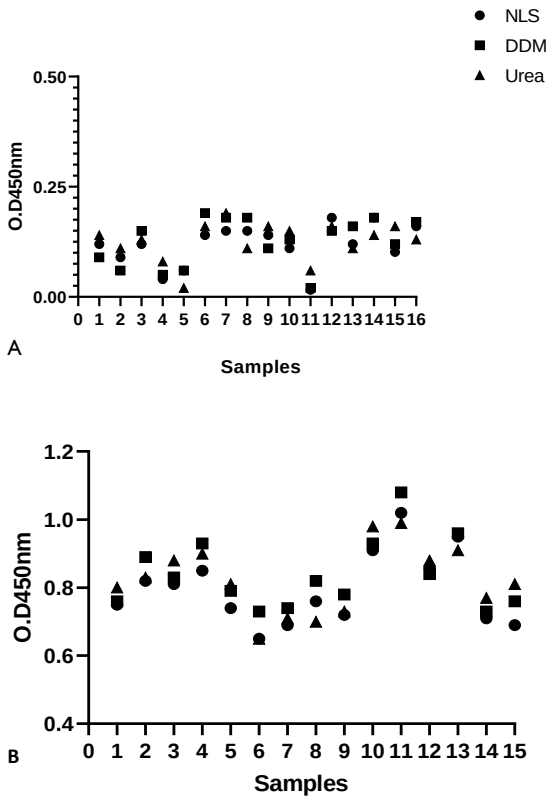

Fig. 4: Analysis of serum samples collected from healthy/vaccinated (A) and infected (B) animals through ELISA based on recombinant proteins purified from Supernatants solubilized in DDM, NLS and urea. "1" indicates the samples NC (A) and PC (B) on the x-axis.

DISCUSSION

General strategy for purifying membrane proteins expressed as inclusion bodies in *E. coli* involves solubilization of IBs using denaturants such as GdnHCl or urea followed by refolding of solublized proteins prior to purification into the bioactive form. Different methods have been identified to solubilize inclusion bodies (misfolded structures) such as 2DR (two-denaturation and refolding) method in which IBs are treated in GdnHCl and then solubilized in urea to dissolve precipitates formed during GdnHCl treatment. Refolding steps were used in both denaturation treatments to enhance the protein yield (Yang *et al.*, 2011). This method is costly and requires huge volume of buffer for the refolding of the target protein. However, protein aggregation and lower protein yields are major bottlenecks of these methods due to the hydrophobic interactions and formation of incorrect disulfide bonds (Vinogradov *et al.*, 2003).

In this study, High level expression of 3ABC protein was achieved using pET-28-SUMO expression vector in *E. coli*. The expression of recombinant 3ABC protein as His_6-SUMO-3ABC was obtained mainly in inclusion bodies. Inclusion bodies harboring 3ABC protein was solubilized in 8M urea and purified using Ni-NTA chromatography. The protein yield obtained from this method is low, most likely due to the protein aggregation (Fig 2B). This attribute was also observed in previous report in which 3ABC protein was solubilized in urea and refolded using dilution (Gelkop *et al.*, 2018). Moreover, it is also reported that 3ABC protein was solubilized in 8M urea, purified and used directly as antigen without using any refolding step (Foord *et al.*, 2007).

Moreover, these insoluble aggregates are reported to possess native secondary conformations and hence, the use of mild solubilization does not completely unfold these native-like structures of protein (Singhvi *et al.*, 2020). In a study, inclusion bodies were treated with mild urea denaturant treatment with trifluoroethanol (TFE) to solubilize the inclusion bodies followed by refolding into bioactive form. TFE is organic solvent which affects secondary and tertiary structures by enhancing the intermolecular interactions and disrupt the hydrophobic interactions. The overall protein yield using this method depends on the efficiency of refolding process. On the other hand, mild solubilization with non-denaturant solubilization agent results into bioactive form of protein without using any refolding process (Upadhyay *et al.*, 2016). Therefore, we used non-denaturant agents such as 0.3% NLS (N-lauryl sarcosine) without using any refolding step that showed the reliable solubilization of

Table 1: Solubilization and purification yield of recombinant 3ABC protein

Detergent solubilized Protein fraction	Detergent solubilization		Purification		
	Supernatant volume	Concentration (mg/mL)	Eluted volume (mL)	Concentration (mg/mL)	Total protein yield (mg)
DDM	20	6.78	5	1.64	8
NLS	20	5.11	5	1.04	5.2
Urea	20	2.54	5	0.65	3.25

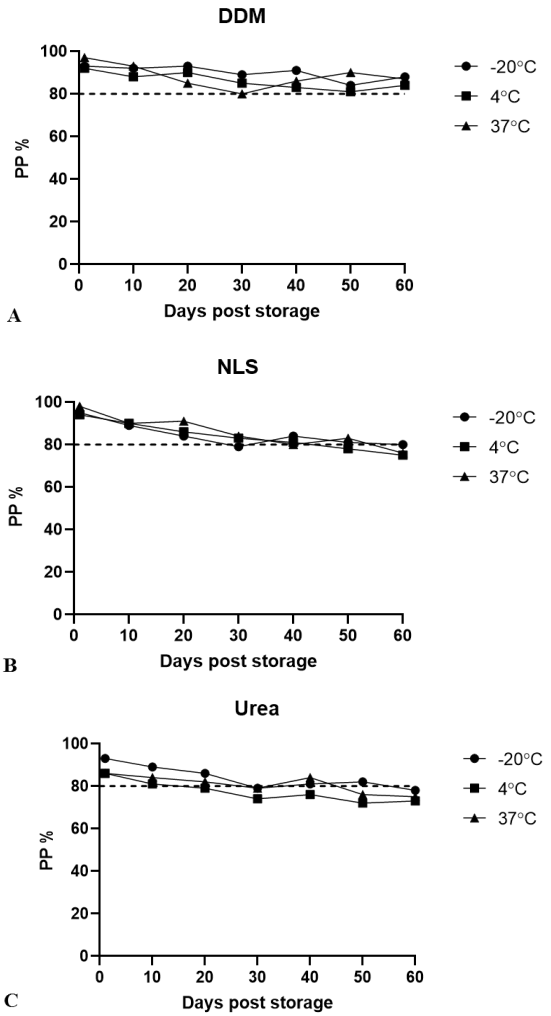

Fig. 5: Thermal stability profile of recombinant 3ABC protein containing DDM (A), NLS (B) and Urea (C) stored at -20ºC, 4ºC and 37ºC.

inclusion bodies and high purification yield of target protein compared with urea solubilization method. This method is suitable for the purification of 3ABC protein (Lu *et al.*, 2007) and many other proteins such as Lys M of tuberculosis (TB) (Mustafa *et al.*, 2019). 3ABC protein was also solubilized in DDM, a non-ionic detergent which significantly improved the purification yield as compared with NLS-solubilization (Table 1). The lower purification yield from NLS-solubilized proteins may be due to the inability of target proteins to nickel based columns owing to masking by NLS. finding was observed in a previous study in which NLS was replaced with a non-ionic detergent to enhance purification of the target protein (Francis *et al.*, 2012).

DDM is a good solubilizing agent, possesses a hydrophilic head group and a hydrophobic acyl tail that allows them in removing the lipids molecules surrounding the membrane protein. Moreover, it consists of favorable properties in order to maintain the functions of mostly aggregation–prone membrane proteins in the solution. A number of proteins have been extracted from inclusion bodies using DDM solubilization method without using any refolding step (He *et al.*, 2014; Kesidis *et al.*, 2020).

The immunoreactivity of 3ABC protein was checked by using it as antigen in ELISA assay. Recombinant 3ABC protein was used at a concentration of 1ug/mL with 1/20 serum dilution as described in previous studies (Lu *et al.*, 2007; Hosamani *et al.*, 2015). All the serum samples showed matching results compared with commercially available kit. The test was considered valid as the O.D. value of positive control obtained from three proteins was three times higher than negative control (Fig 4).

Membrane proteins show insolubility in aqueous solutions and poor stability in detergents which makes them difficult for functional analysis. Temperature variations occur during the manufacturing, storage, and transportation which degrade the less thermally stable proteins and makes them to lose their potency (Carlson *et al.*, 2018). Therefore, thermal stability is a critical quality parameter for the characterization during the discovery and development of the proteins. Furthermore, the analysis of thermal unfolding of protein is also essential to illuminate the thermodynamic stability and unfolding pathways of the protein (Bowie, 2001). Hence, we checked the stability of each solubilized protein at different temperatures (Fig 5). This aspect for 3ABC protein is not reported so far probably due to the solubilization of membrane proteins often results in the poor stability of the protein, protein unfolding and subsequent protein aggregation. Previously, the thermal stability of 2C (Mahajan *et al.*, 2013) and 3AB (Mohapatra *et al.*, 2011) has been reported which showed good stability rates when stored at different temperatures in comparison with storage at -70ºC since both proteins were produced soluble in *E. coli.*

In summary, this was an effective and reliable method using non-ionic detergent for the purification of 3ABC polyprotein from the inclusion bodies in higher yields and soluble form. This purification strategy can be applied without using any renaturation step while maintaining the stability of the protein for subsequent applications.

Conclusions: The mild solubilization of inclusion bodies with non-ionic detergent is found reliable and efficient method to purify 3ABC protein without using any refolding procedures. Significant differences in the purification yields of the protein from inclusion bodies with other methods i.e. ionic detergent and high chaotropic denaturant, confirms the choice of non-ionic detergent for solubilization and purification of proteins is authentic. The recombinant protein showed reliable performance on ELISA and good thermal stability as well.

Acknowledgements: The study was supported by IAEA TCP 5050 project to MH. We are thankful to Mr. Hafiz Muhammad Muzammal, Mr. Irfanullah, Mr. Asad Javed and Mrs. Shaista for providing technical assistance in the lab during the study.

Authors contribution: MAZ, conducted experimentation, data analysis and writing the manuscript. MS helped in data analysis, data correction and writing and reviewing the manuscript. MH conceived the research, data curation and reviewed the manuscript.

REFERENCES

Barrera FN, Renart ML, Molina ML, *et al*., 2005. Unfolding and refolding in vitro of a tetrameric, α-helical membrane protein: the prokaryotic potassium channel KcsA. Biochemistry 44:14344-52.

Bowie JU, 2001. Stabilizing membrane proteins. Curr Opin Struct Biol 11:397-402.

Carlson ML, Young JW, Zhao Z, *et al*., 2018. The Peptidisc, a simple method for stabilizing membrane proteins in detergent-free solution. Elife 7:e34085.

Foord AJ, Muller JD, Yu M, *et al*., 2007. Production and application of recombinant antibodies to foot-and-mouth disease virus non-structural protein 3ABC. J Immunol Methods 321:142-51.

Francis VG, Majeed MA and Gummadi SN, 2012. Recovery of functionally active recombinant human phospholipid scramblase 1 from inclusion bodies using N-lauroyl sarcosine. J Ind Microbiol Biotechnol 39:1041-8.

Gao Y, Sun SQ and Guo HC, 2016. Biological function of Foot-and-mouth disease virus non-structural proteins and non-coding elements. Virol J 13:1-17.

Gelkop S, Sobarzo A, Brangel P, *et al*., 2018. The development and validation of a novel nanobody-based competitive ELISA for the detection of foot and mouth disease 3ABC antibodies in cattle. Front Vet Sci 5:250.

He Y, Wang K and Yan N, 2014. The recombinant expression systems for structure determination of eukaryotic membrane proteins. Protein Cell 5:658-72.

Hosamani M, Basagoudanavar S, Selvan RT, *et al*., 2015. A multi-species indirect ELISA for detection of non-structural protein 3ABC specific antibodies to foot-and-mouth disease virus. Arch Virol 160:937-44.

Kaur J, Kumar A and Kaur J, 2018. Strategies for optimization of heterologous protein expression in E. coli: Roadblocks and reinforcements. Int J Biol Macromol 106:803-22.

Kesidis A, Depping P, Lodé A, *et al*., 2020. Expression of eukaryotic membrane proteins in eukaryotic and prokaryotic hosts. Methods 180:3-18.

Kruger NJ, 2009. The Bradford method for protein quantitation In: The protein protocols handbook: 3rd Ed: Agent (Walker JM, ed). Humana Press, New York, USA pp:17-24.

Lu Z, Cao Y, Guo J, *et al*., 2007. Development and validation of a 3ABC indirect ELISA for differentiation of foot-and-mouth disease virus infected from vaccinated animals. Vet Microbiol 125:157-69.

Mahajan S, Mohapatra JK, Pandey LK, *et al*., 2013. Truncated recombinant non-structural protein 2C-based indirect ELISA for FMD sero-surveillance. J Virol Methods 193:405-14.

Mason PW, Grubman MJ and Baxt B, 2003. Molecular basis of pathogenesis of FMDV. Virus Res 91:9-32.

Mohapatra JK, Pandey LK, Sanyal A, *et al*., 2011. Recombinant non-structural polyprotein 3AB-based serodiagnostic strategy for FMD surveillance in bovines irrespective of vaccination. J Virol Methods 177:184-92.

Mustafa AD, Kalyanasundram J, Sabidi S, *et al*., 2019. Recovery of recombinant Mycobacterium tuberculosis antigens fused with cell wall-anchoring motif (LysM) from inclusion bodies using non-denaturing reagent (N-laurylsarcosine). BMC Biotechnol 19:1-11.

Rudolph R and Lilie H, 1996. In vitro folding of inclusion body proteins. FASEB J 10:49-56.

Salem R, El-Kholy AA, Waly FR, *et al*., 2021. Removal of 3C protease from the 3ABC improves expression, solubility, and purification of the recombinant 3AB of foot-and-mouth disease virus. Virus Genes 57:72-82.

Schramm FD, Schroeder K and Jonas K, 2020. Protein aggregation in bacteria. FEMS Microbiol Rev 44:54-72.

Singh SM and Panda AK, 2005. Solubilization and refolding of bacterial inclusion body proteins. J Biosci Bioeng. 99:303-10.

Singhvi P, Saneja A, Srichandan S, *et al*., 2020. Bacterial inclusion bodies: a treasure trove of bioactive proteins. Trends Biotechnol 38:474-86.

Song HS, Lee SH, Oh EH, *et al*., 2009. Expression, solubilization and purification of a human olfactory receptor from Escherichia coli. Curr Microbiol 59:309-14.

Srisombundit V, Tungthumniyom N, Linchongsubongkoch W, *et al*., 2013. Development of an inactivated 3Cpro-3ABC (mu3ABC) ELISA to differentiate cattle infected with foot and mouth disease virus from vaccinated cattle. J Virol Methods 188:161-7.

Tewari A, Ambrose H, Parekh K, *et al*., 2021. Development and Validation of Confirmatory Foot-and-Mouth Disease Virus Antibody ELISAs to Identify Infected Animals in Vaccinated Populations. Viruses 13:914.

Tripathi NK and Shrivastava A, 2019. Recent developments in bioprocessing of recombinant proteins: expression hosts and process development. Front Bioeng Biotechnol 7:420.

Tsumoto K, Ejima D, Kumagai I, *et al*., 2003. Practical considerations in refolding proteins from inclusion bodies. Protein Expr Purif 28:1-8.

Upadhyay V, Singh A, Jha D, *et al*., 2016. Recovery of bioactive protein from bacterial inclusion bodies using trifluoroethanol as solubilization agent. Microbial Cell Factories 15:1-13.

Vinogradov AA, Kudryashova EV, Levashov AV, *et al*., 2003. Solubilization and refolding of inclusion body proteins in reverse micelles. Anal Biochem 320:234-8.

Wang L, Zhou H, Li Z, *et al*., 2015. One-step extraction of functional recombinant aquaporin Z from inclusion bodies with optimal detergent. Protein Expr Purif 115:146-52.

Yang Z, Zhang L, Zhang Y, *et al*., 2011. Highly efficient production of soluble proteins from insoluble inclusion bodies by a two-step-denaturing and refolding method. PloS One 6:e22981.

Zeedan GSG, Abdalhamed AM, Farag TK, *et al*., 2020. Optimization of serological diagnostic methods for rapid field detection of foot-and-mouth disease virus antigen and antibodies in natural infected bovine specimens. Int J Vet Sci 9:30-5.