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Improvement of the purification of Saint Louis encephalitis virus NS2B-NS3 recombinant protease expressed in *Escherichia coli*

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ABSTRACT

The NS2B-NS3 serine protease of Saint Louis Encephalitis virus (SLEV), a potential target for antiviral drug design, has been over-expressed as a recombinant His-tag protein in *Escherichia coli* for future structural determination. The production process resulted in a soluble protease with co-purification of DnaK, a bacterial molecular chaperone already described in *E. coli* protein expression. Two approaches were tested to remove this specific contaminant. The fusion protein bound to the purification resin was washed with MgATP plus soluble denatured *E. coli* proteins before elution, but this method proved to be poorly efficient due to a substantial loss of the targeted recombinant protease. After the immobilized metal affinity chromatography step, the use of gel permeation chromatography with addition of arginine in the mobile phase led to effective separation of the native viral protease from the DnaK aggregates. By this way, SLEV ΔNS2B-NS3pro protease was purified as a functional protein with a purity greater than 90% suitable for crystallization attempts.

1. Introduction

The genus *Flavivirus*, family *Flaviviridae*, comprises more than 70 viruses [1]. Many of them, such as Dengue viruses (DENV), Yellow fever virus (YFV), West-Nile virus (WNV) or Tick-borne encephalitis virus (TBEV), are important human pathogens [2]. Few vaccines and no specific antiviral drugs are available [3] in spite of active research due to their large distribution and the potential severity of infections [4,5].

Among the flavivirus-encoded enzymes whose activities are known to be crucial for the viral replication cycle, the NS2B-NS3 protease complex has been widely studied [6]. Indeed, this complex, also known as "flavivirin" [7], plays a key role in post-translational processing of the viral polyprotein by mediating several cleavages [8,9]. Therefore, it is considered as a promising target for antiviral therapy [10,11]. In this complex, the ~180 amino acid long N-terminal domain of NS3, called NS3pro, contains the conserved catalytic triad of trypsin-like serine proteases [12,13], whereas NS2B is a membrane-anchored protein required as a co-factor [14,15].

In previous works, we have studied the protease complex of different flaviviruses expressed as hexahistidine-tagged recombinant proteins [16–18]. These constructions consisted of NS3pro domain covalently fused with the central hydrophilic domain of NS2B (ΔNS2B), forming a complex with protease activity [14,19]. After expression in *Escherichia coli* and purification by immobilized-metal ion affinity chromatography (IMAC), these proteins were active towards chromogenic and fluorogenic substrates. However, despite the purification step, the recovered proteases were found contaminated by additional proteins of bacterial origin. These unwanted proteins did not exhibit any proteolytic activity which could disturb the protease assays but their presence made difficult other studies requiring highly pure recombinant proteins, such as crystallographic studies. After optimisation of the production process, the major contaminant identified in the purified SLEV pro-

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tease fractions was the bacterial molecular chaperone DnaK. As already described [20], this unwanted protein was not eliminated by standard ion exchange or gel permeation chromatographies (GPC), consistent with strong protein–protein interaction.

Herein, we tested and compared two approaches to remove this DnaK contamination from soluble SLEV protease (SLEV ΔNS2B-NS3pro) after over-expression in *E. coli*. The first method was based on washing the fusion protein bound to the purification resin with MgATP plus soluble denatured *E. coli* proteins before elution. The second method consisted of a two-step chromatographic process including a GPC with addition of 0.5 M arginine to the mobile phase. Using this two-step process, we reached a high purity of the protease, up to 90%, suitable for crystallization experiments. The described purification method improves the separation from soluble bacterial contaminants preserving the native functional structure of the expressed protein. It could have application to a broad spectrum of recombinant proteins.

2. Materials and methods

2.1. Cloning and expression

The SLEV ΔNS2B-NS3pro protease construction used in this study has been already described [17]. Expression of the soluble protease was optimal under following conditions: competent *E. coli* BL21-CodonPlus (DE3)-RIL (Stratagene, Amsterdam, The Netherlands) transformed with the expression plasmid pET-DEST42 (Invitrogen, Cergy Pontoise, France) were grown in TB medium (Interchim, Montluçon, France) containing tetracycline (10 μg mL^{-1}), chloramphenicol (75 μg mL^{-1}) and ampicillin (100 μg mL^{-1}) at 37 °C until the OD_{600} reached 0.8. Recombinant protein expression was induced with isopropyl-β-D-thiogalactopyranoside 1 mM (Sigma–Aldrich Chimie, Saint-Quentin Fallavier, France) for 6 h at 22 °C, 220 rpm. Cells were harvested by centrifugation at 4000 × *g*, 30 min, 4 °C and stored at −80 °C until use. A 1.5 L culture resulted in about 13 g of wet cells.

2.2. Lysis

Cell pellets were thawed and resuspended in 1 mL of lysis buffer (Hepes 50 mM pH 7.5, NaCl 300 mM, glycerol 5%)/20 mL of original culture. The resuspended cells were disrupted by sonication (36 × 5 s pulses at 50 W) on ice and then recentrifuged for 30 min at 27,000 × *g*, 4 °C. The supernatant containing the soluble SLEV protease was immediately purified.

2.3. Purification

Chromatographic steps were achieved in a cold room at 4 °C using an ÄKTA$_{FPLC}$ system (Amersham Biosciences, Orsay, France).

2.3.1. Immobilized affinity chromatography

The supernatant was mixed in batch mode with 6 mL of Ni-NTA Superflow resin (Qiagen, Courtabœuf, France) on a rotary shaker at 4 °C for 90 min. The lysate-Ni-NTA batch was packed onto a XK16/20 column (Amersham Biosciences). Imidazole gradient was made by mixing two separate buffers: Hepes 50 mM pH 7.5, NaCl 300 mM (Buffer A) and Hepes 50 mM pH 7.5, NaCl 10 mM, imidazole 100 mM (Buffer B). The column was washed at a flow rate of 2 mL min^{-1} with a buffer containing 20 mM imidazole (80% Buffer A, 20% Buffer B) until the OD_{280} returned to baseline. The protein was then directly eluted with a buffer containing 100 mM imidazole (100% Buffer B) or the resin was again washed with ten bed volumes (50 mL) of buffer A containing 20 mM imidazole, 5 mM MgATP plus 0.1 mg mL^{-1} denatured *E. coli* proteins before elution. The purified collected fractions were concentrated up to 3 mL using Vivaspin 20 concentrators (Vivascience AG, Hannover, Germany) with a 10 kDa cut-off membrane and stored at −80 °C in 5% (v/v) glycerol.

2.3.2. Gel permeation chromatography

Gel permeation chromatography was carried out on a Superdex-75 prep grade column (XK 26/60, Amersham Biosciences) equilibrated with Buffer C (20 mM TrisHCl pH 8.0, 100 mM NaCl, L-arginine 0.5 M). Chromatography was performed after incubation of the IMAC-purified proteins for 1 h at 4 °C in Buffer C. Samples were then concentrated to a volume of 2.5 mL before loading. Fractions of 2 mL were collected at a flow rate of 1 mL min^{-1} and absorbance was measured at 280 nm.

2.4. SDS-PAGE, Western blot and bioanalyzer analysis

Collected fractions were analysed by Coomassie blue staining after SDS-PAGE. For western blot analysis, gels were transferred to a PVDF membrane (Amersham) by semidry blotting. Anti-V5-HRP monoclonal antibodies (Invitrogen) diluted 1/5000 were used to detect the SLEV ΔNS2B-NS3pro protein after addition of 3,3′,5,5′-tetramethylbenzidine (TMB, Promega).

All samples were also analysed using the Agilent 2100 and the Protein 200 LabChip kit Bioanalyzer (Agilent Technologies) following the manufacturer's instructions.

2.5. Quantitation and purity level determination

Crude protein concentrations were measured using the BCA Protein Assay kit with BSA as standard (Pierce, Perbio Science, Brebieres, France). The purified SLEV ΔNS2B-NS3pro protein and the contaminant were quantified on electrophoregrams by comparison with the internal standard (Protein 200 LabChip kit, Agilent Technologies). The degree of purity was also determined using the Agilent 2100 bioanalyzer and the Protein 200 LabChip kit.

2.6. Enzymatic activity

After purification, the protease activity of protein batches was measured using *t*-butyloxycarbonyl-glycyl-L-lysyl-L-arginine 4-methylcoumaryl-7-amide (Boc-GKR-MCA, Orpegen Pharma, Heidelberg, Germany) as fluorogenic substrate, following the experimental procedures previously described [17].

2.7. Mass spectrometry analysis

For identification, the proteins were spotted, excised from the gel and processed to obtain tryptic peptides as already reported [20]. An Ettan MALDI-TOF/Pro mass spectrometer (Amersham Biosciences) was used to obtain peptide mass fingerprinting (PMF). PMF database searching was carried out using MASCOT (http://www.matrixscience.com/) on-line available software. The identity of proteins was annotated using the SWISS-PROT and TrEMBL database.

3. Results and discussion

3.1. Expression of recombinant SLEV ΔNS2B-NS3pro protease and identification of the bacterial contaminant

Previous experiments showed that the SLEV protease was found mainly in insoluble inclusion bodies [17]. A new production protocol, using lower growth temperature (22 °C) for 6 h after induction, gave high expression levels of the soluble 28 kDa protein. However, when SLEV ΔNS2B-NS3pro protease was expressed in *E. coli* and

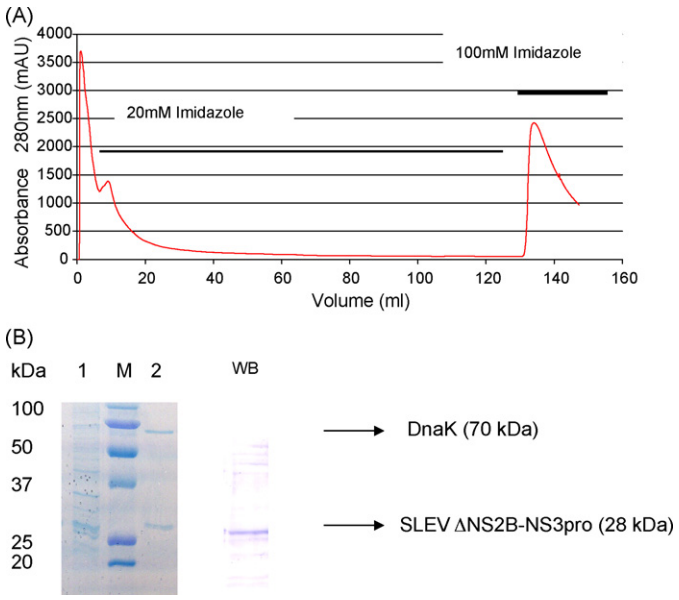

Fig. 1. Metal chelate affinity chromatogram (A) and SDS-PAGE, Western blotting analysis (B) of SLEV ΔNS2B-NS3pro protein from Ni^{2+} column. (A) After centrifugation for 30 min at 27,000 × *g*, 4 °C, the soluble fraction from 1.5 L of bacterial lysate was loaded onto Ni^{2+} affinity column then washed and eluted with different concentrations of imidazole. (B) SDS-PAGE analysis after coomassie Blue staining: lane M, molecular weight markers; lane 1, the crude lysate; lane 2, the elution fraction at 100 mM imidazole. WB: Western blot analysis of the 100 mM imidazole eluted fraction using anti-V5-HRP monoclonal antibodies to detect the SLEV NS2B-NS3pro protein.

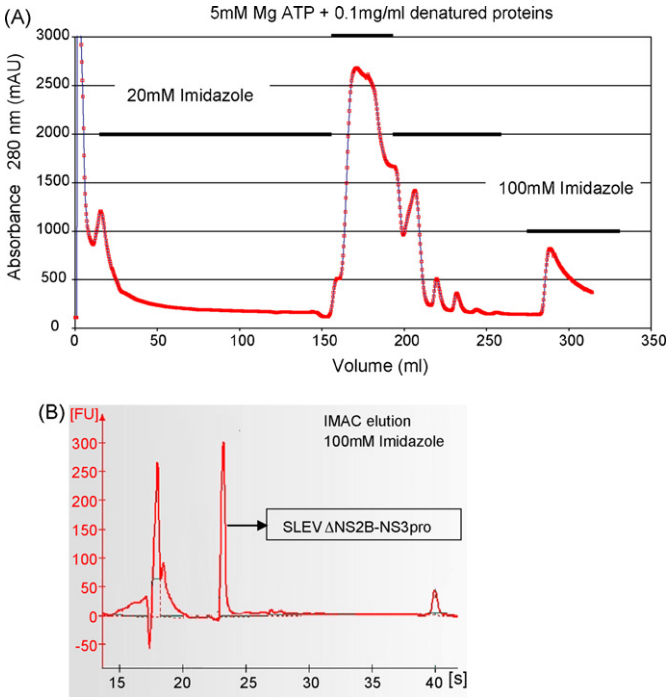

Fig. 2. Metal chelate affinity chromatogram (A) and electropherogram (B) of SLEV ΔNS2B-NS3pro eluted fraction from Ni^{2+} column washed with MgATP plus soluble denatured *E. coli* proteins. (A) After centrifugation for 30 min at 27,000 × *g*, 4 °C, the soluble fraction from 1.5 L of bacterial lysate was loaded onto Ni^{2+} affinity column and washed with 50 mL of a buffer containing 5 mM MgATP plus 0.1 mg/mL denatured *E. coli* proteins before elution with 100 mM imidazole. (B) Eluted sample was then analysed using the Agilent 2100 Bioanalyser.

purified by IMAC (Fig. 1A), co-purification of a ~70 kDa protein was observed (Fig. 1B). Attempt to remove this unwanted 70 kDa protein by using anion exchange chromatography (MonoQ 5/50, GE Healthcare) or gel permeation chromatography (Superdex 200HR 10/30, Amersham Biosciences) were unsuccessful (data not shown). These results suggested a strong protein–protein interaction with this main contaminant which was identified by mass spectrometry analysis as the bacterial molecular chaperone DnaK (molecular weight 71,378 Da, number Swiss-Prot/TrEMBL: HTPG_ECO57, 34% sequence coverage with 16 peptides matched) already described [21,22].

Other low molecular weight bands ~25 kDa detected in SDS-PAGE or Western blot analysis (Fig. 1B) were attributed to autocatalytic cleavages of the SLEV ΔNS2B-NS3pro protease as previously reported when recombinant flaviviral proteases are over-expressed under native conditions [16–18,23–28].

3.2. IMAC purification and MgATP washing

Advances in the study of viral proteases have been dependent on the ability to produce significant quantities of pure enzymes. *E. coli* is by far the most used expression system with success for viruses of the *Flavivirus* genus where crystal structures of the NS2B-NS3 protease complex have been reported for DENV [29,30], WNV [30], and Murray Valley encephalitis virus [31]. For large-scale purification of recombinant proteins, IMAC is largely used due to the production of a pure protein sample through a single purification step. Although the interactions of the histidine tag with the affinity material are highly specific, contaminating proteins can bind non-specifically to the beads or to the tagged protein resulting in unwanted impurities co-purification [32]. In fact, they are mainly native *E. coli* proteins exhibiting a high affinity for divalent cations or histidine-tagged proteins. The presence of abundant co-purified bacterial proteins such as Hsp70 molecular chaperone homolog, have also been observed [20–22].

In our system, the over-expressed SLEV ΔNS2B-NS3pro protease was strongly associated with the bacterial DnaK protein. High salt concentrations or addition of 10–50 mM imidazole during the IMAC wash steps failed to improve the purification. This specific interaction may be explained by the chaperone affinity for the connector peptide region of pET fusion vectors [21]. Another explanation could be the DnaK binding to partially unfolded polypeptide region of the over-expressed protease. In case of protein misfolding, it is known that substrate binding and release from DnaK are coupled to its ATPase activity and some methods have been developed to remove this protein association [21,22]. However, in our hands, washing the fusion protein bound to the IMAC resin with MgATP plus soluble denatured *E. coli* proteins (Fig. 2) was poorly efficient. This step resulted in a substantial loss of the recombinant protein (about eight-fold) in spite of a purity improvement (71–85%) (Table 1). Similar negative results have been previously reported for the purification of the Chikungunya virus nsP2 protease contaminated with the same bacterial DnaK protein [20].

3.3. GPC purification with arginine additive

Gel permeation chromatography (GPC) is one of many versatile protein separation techniques based on the hydrodynamic radius of macro-molecules. In flavivirus enzyme structure studies, this chromatography has been frequently used after IMAC to obtain pure recombinant proteins suitable for crystallization assays [29–31]. To reduce non-specific binding to the stationary phase during GPC, arginine has been proven to be an efficient additive [33]. Since arginine is a non-toxic common amino acid that can be readily removed, it was successfully used during many purification

Table 1
Purification of SLEV ΔNS2B-NS3pro protease from *E. coli* extract (1.5 L of culture, 13 g wet cells)

Fractions	%SLEV ΔNS2B-NS3pro[a]	%DnaK[a]	Total protein[b] (mg)	Total protease activity[c] (URF/min)	Total protease activity per mg protein (URF/min/mg)
IMAC elution after MgATP wash	85	10	8	450 ± 8	56.25
IMAC elution	71	23	64.5 (**52**)	3570 ± 36	55.35
GPCpeak1	28	65	4.2	55 ± 3	13.09
GPCpeak2	90	6	32.3	2090 ± 23	64.7

[a] Protein purity determined with the protein 200 LabChip kit (Agilent Technologies).
[b] Protein quantitation by BCA assay. Value in bold correspond to the amount of total protein obtained after concentration and injected in GPC.
[c] Protease activity was determined using Boc-GKR-MCA as substrate and measurements were performed in triplicate for each data point.

processes [34,35] like antibody purification [36] or hydrophobic interaction chromatography [37] by dissociating strong molecular interactions without potential denaturation.

Taking these results into account, the effect of arginine on the SLEV ΔNS2B-NS3pro protease purification process was evaluated. Due to incompatibility of arginine with Ni-NTA matrices, this additive could not be included during the affinity chromatography step. Moreover, to avoid putative column performance decrease or protein structure modification, the arginine was assayed at a commonly used 0.5 M concentration. After contact for 1 h at 4 °C with buffer containing 0.5 M arginine, the IMAC-purified protease was concentrated and then loaded on a Superdex-75 prep grade column for GPC purification using aqueous arginine solution in the mobile phase. The separation profile showed the appearance of a minor peak followed by a major one (Fig. 3A). The total peak area was about 70% of the applied amount (52 mg of total protein loaded *versus* 37 mg of total protein eluted) (Table 1). SDS-PAGE and Agilent 2100 analysis of the two peaks indicated an effective separation of the SLEV ΔNS2B-NS3pro protease from its DnaK contaminant (Figs. 3B and 4B). The minor peak contained about 65% of the unwanted bacterial aggregate as shown in Fig. 4A. Finally, this GPC increased the protease purity of about 20% to reach 90% when compared with the IMAC step alone (Fig. 4A).

In contrast, GPC purification of the same protease fraction without arginine supplement led to a lower protein recovery rate of about 50%. This result indicated that some amounts of the protein tended to stick to the column matrix [33]. Moreover, the unique GPC peak obtained was analysed and showed no effective dissociation of the SLEV protease to its main DnaK aggregate (data not shown).

3.4. Protease activity analysis of the purified fractions

Generally, aggregation suppressors or strong separative agents are denaturant additives which destabilize the native protein structures. In contrast arginine has been shown effective in protein solubilization, aggregation suppression or dissociation. It is used in several column chromatography protocols without adverse effect on the purified protein [34,38]. Since arginine

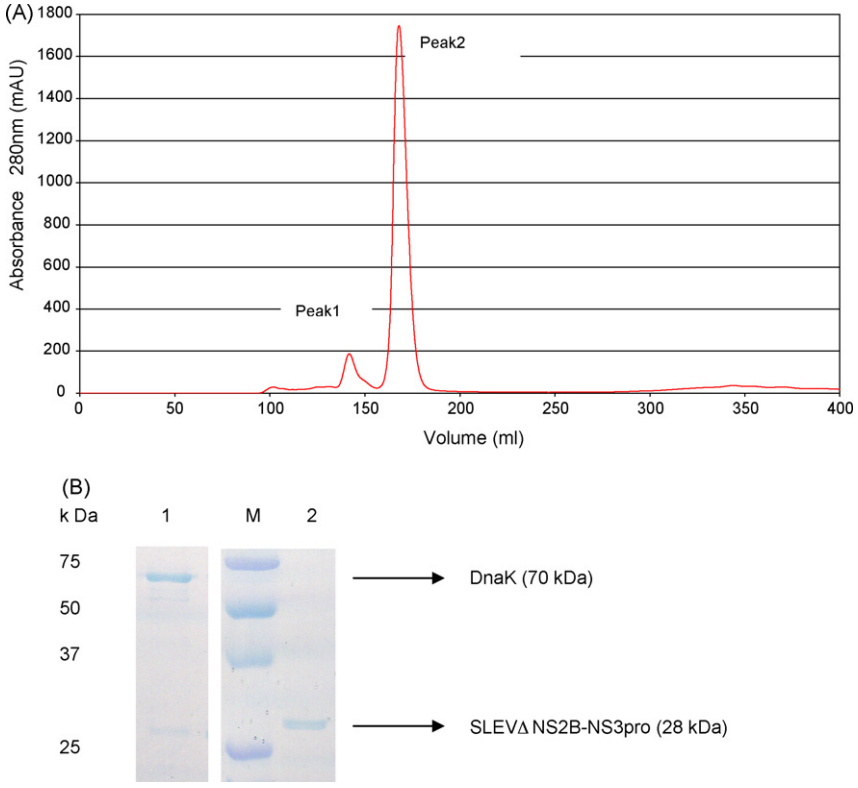

Fig. 3. GPC profile (A) and SDS-PAGE (B) analysis of SLEV ΔNS2B-NS3pro fusion protein from Superdex-75 prep grade column. (A) Chromatography was performed after incubation of the IMAC-purified proteins for 1 h at 4 °C in Buffer C. Fractions of 2 mL were collected at a flow rate of 1 mL min^{-1} in the elution Buffer C and absorbance was measured at 280 nm. (B) SDS-PAGE analysis after Coomassie Blue staining: lane M, molecular weight markers; lane 1, peak1 fraction; lane 2, peak2 fraction.

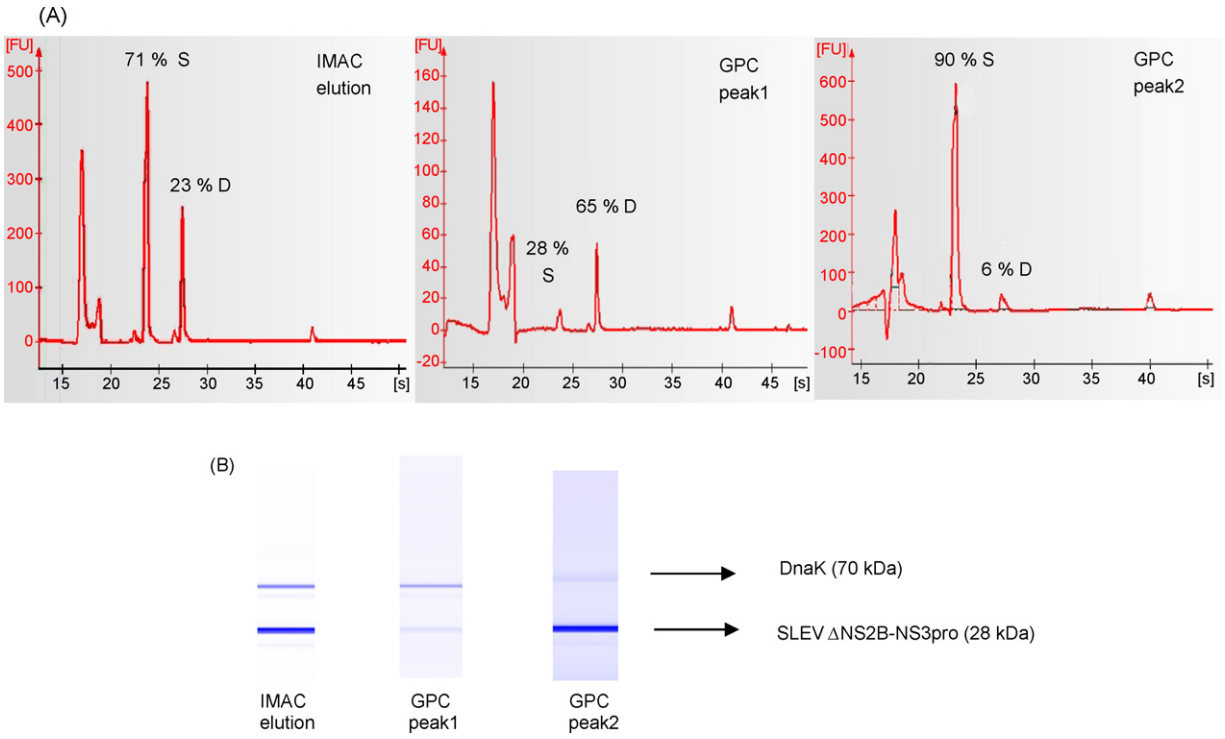

Fig. 4. Electrophoregrams of the IMAC and GPC-purified fractions using the Agilent 2100 Bioanalyser. (A) Analysis were performed using the Protein 200 LabChip kit Bioanalyzer (Agilent Technologies). The electrophoregrams visualize the separation of the proteins according to their molecular weight (kDa) and the relative protein concentration were determined based on measuring peak areas and comparison to internal standard with known protein concentration. S: SLEV ΔNS2B-NS3pro, D: DnaK. (B) Gel-like image corresponding to each electrophoregram.

improves purification in native conditions, protein separation methods in the presence of such additive are useful techniques.

To evaluate the arginine impact on our SLEV-purified samples, enzymatic assays were performed under the experimental procedures previously described [17]. In the presence of 50 mM arginine, the purified protease showed a high 85% relative proteolytic activity which suggested no adverse effect on the enzyme structure (data not shown). The slight activity decrease could be attributed to the known competitive inhibitory effect of the arginine on trypsin-like serine proteinases. As already observed [38,39], it seems that the addition of 0.5 M arginine (50 mM final concentration in our enzymatic assays) resulted in very little effect on our protein structure. Thus, it was possible and convenient to follow the SLEV protease purification steps by measuring the proteolytic activity on successive fractions (Table 1).

These results demonstrated the possibility to purify recombinant enzymes after solubilization or elution by arginine-containing solvents without its removing. Arginine also enhanced correct structure and stabilized native conformation [38] that might be suitable for subsequent crystallization experiments. In the other hand, our data suggested that removing DnaK from fusion proteins did not impact either the amount of recovered proteins or their enzymatic activities.

4. Conclusion

Many proteins crystallize readily and under multiple conditions even after relatively crude purifications, whereas others may only produce crystals in a very narrow 'window'. However, for the DENV NS2B-NS3 protease complex, very pure sample preparation was a prerequisite for identifying crystallization and obtaining X-ray-quality crystals [30].

In this study, an efficient approach was developed to remove a DnaK contamination in Saint Louis encephalitis virus NS2B-NS3 protease over-expressed in *E. coli*. The purification was improved under native conditions by using arginine in a GPC step allowing suppression of unwanted specific inter-molecular interactions and separation of "sticky" proteins without potential denaturation. Finally by two purification steps, the Saint Louis encephalitis virus NS2B-NS3 protease purity reached 90%. Moreover, the proteolytic activity was retained without arginine removing which might be convenient for future crystallographic studies.

This method could also be easily applied to any His-tag fused recombinant protein for improving dissociation from specific bacterial aggregates.

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