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Effects of Lower Temperature on Expression and Biochemical Characteristics of HCV NS3 Antigen Recombinant Protein

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Abstract: The nonstructural antigen protein 3 of the hepatitis C virus (HCV NS3), commonly-used for HCV ELISA diagnosis, possesses protease and helicase activities. To prevent auto-degradation, a truncated NS3 protein was designed by removing the protease domain. Firstly, it was overexpressed in *E. coli* by IPTG induction under two different temperatures (25 and 37 °C), and purified using affinity chromatography to attain homogeneity above 90%. The molecular mass of purified protein was determined to be approx. 55 kDa. While lowering the temperature from 37 to 25 °C, the yield of the soluble fraction of HCV NS3 was increased from 4.15 to 11.1 mgL^{-1} culture, which also improved the antigenic activity and specificity. The protein stability was investigated after long-term storage (for 6 months at −20 °C) revealed no loss of activity, specificity, or antigenic efficacy. A thermal stability study on both freshly produced and stored HCV NS3 fractions at both temperatures showed that the unfolding curve profile properly obey the three-state unfolding mechanism. In the first transition phase, the midpoints of the thermal denaturation of fresh NS3 produced at 37 °C and 25 °C, and that produced after long-term storage at 37 °C and 25 °C, were 59.7 °C, 59.1 °C, 55.5 °C, and 57.8 °C, respectively. Microplates coated with the fresh NS3 produced at 25 °C or at 37 °C that were used for the HCV ELISA test and the diagnosis outcome were compared with two commercial kits—Abbott HCV EIA 2.0 and Ortho HCV EIA 3.0. Results indicated that the specificity of the HCV NS3 produced fresh at 25 °C was higher than that of the fresh one at 37 °C, hence showing potential for application in HCV ELISA diagnosis.

Keywords: HCV; NS3; protein expression; diagnosis; helicase; protease

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1. Introduction

The hepatitis C virus (HCV), which infects approximately 3% of the world population annually, is a major etiology of the blood transfusion-associated non-A and non-B hepatitis [1,2]. The reduction in post-transfusion HCV incidence for blood donors largely depends on proper execution and lab practice. The most common screening method currently used employs ELISA (enzyme-linked immunosorbent assay) or NAT (nucleic acid amplification technology) to detect anti-HCV antibodies or HCV RNA in the serum sample. Although NAT could identify an extremely low level of virus at a very early stage of the infection, it is time-consuming, and the contamination potential limits its clinical application. ELISA is hence still a more favorable choice for its relatively cheap and rapid output [3].

HCV, which belongs to the Flaviviridae family, is a small-enveloped virus with a single-stranded sense (positive) RNA genome [4]. The viral genome encodes a single polyprotein,

which is composed of about 3000 amino acids [5]. This polyprotein is cleaved by viral and cellular proteases into several mature proteins, which includes three structural proteins (core protein, envelopes 1 and 2) and six nonstructural proteins (NS2, NS3, NS4a, NS4b, NS5a, NS5b). Among these mature proteins, only a few are immunogenic [6,7], whereas among nonstructural proteins, protein 3 (NS3) has been demonstrated to possess potential antigenic efficacy and be able to induce high levels of antibodies in the early infection stage [8]. It also possesses enzymatic activities, including protease [9] and helicase [10], which are required for viral replication. Mainly due to its conserved sequence, apart from its immunogenic property, NS3 has been commonly used as an antigen of several commercial ELISA diagnostic products (MUREX, MP, ORTHO, INNOTEST, GBC) [11–13]. Several studies have been carried out to produce soluble NS3 fractions in high quantities through the cloning approach, including the refolding of overexpressed bulk fractions as inclusion bodies of NS3 recombinant proteins [14,15], and to produce an active domain such as the protease domain [16] or the helicase domain [17]. Nevertheless, due to its auto-protease activity and its aggregation in the *Escherichia coli* expression system, the industrial-scale production of this recombinant protein is still a major challenge.

Therefore, in the current study, a clone specially designed to synthesize a truncated NS3 recombinant protein (without the protease domain) when overexpressed in the *E. coli* expression system was developed. The aim of this research is to overcome the refolding challenge of the recombinant protein (as inclusion body: lacking proper folding and activity) when expressed under a strong promoter in the *E. coli* system growing at 37 °C. The refolding of the inclusion body undergoes several time-consuming tedious steps of high salt solubilization and salt removing purification. Alternatively, to reduce the rate of protein expression (to avoid inclusion body formation) and improve its folding (soluble fraction), a lower temperature (25 °C) culture incubation was opted for its expression besides the usual temperature incubation at 37 °C. Temperature effects on recombinant protein production, protein stability, and antigenicity were characterized, and a comparative account was presented between both recombinant proteins obtained from the 25 °C and 37 °C incubations. The comparison of recombinant HCV ELISA with two commercial kits, the Abbott HCV EIA 2.0 and the Ortho HCV EIA 3.0, were also carried out and thoroughly discussed.

2. Results and Discussions

2.1. Preparation of Recombinant HCV NS3

2.1.1. Expression and Purification of the Recombinant NS3 Proteins

To prevent auto-degradation, a recombinant clone encoding a truncated NS3 with partial protease domain deletion was generated. The recombinant clone was overexpressed in the *E. coli* expression system by IPTG induction at 37 °C or 25 °C, and then the *E. coli* cell was collected. After cell disruption, about 15% of the NS3 protein was present in the cell debris (data not shown) of the total culture biomass, and only the soluble NS3 protein present in the supernatant fraction was used for purification. As shown in Figure 1, the bands for the NS3 protein in the supernatant fraction were more abundant at the 25 °C than at the 37 °C incubations. When chromatography was applied, both 37 °C and 25 °C incubated NS3 could be isolated efficiently by a single Talon affinity column, as shown in Figure 2A. Finally, as listed in Table 1, the NS3 production yield of the 25 °C incubation was three-fold higher than that of the 37 °C incubation. Even though the incubation at lower temperatures was designed to extend the incubation time from 4 to 18 h, the production yield was elevated dramatically from 4.15 to 11.1 mg/L. It was determined that when the incubation temperature was reduced from 37 to 25 °C, the growth rate of bacteria was also reduced; however, the lower temperature conditions significantly increased the 3D folding degree of NS3 towards proper protein structure and further promoted the protein's solubility and productivity.

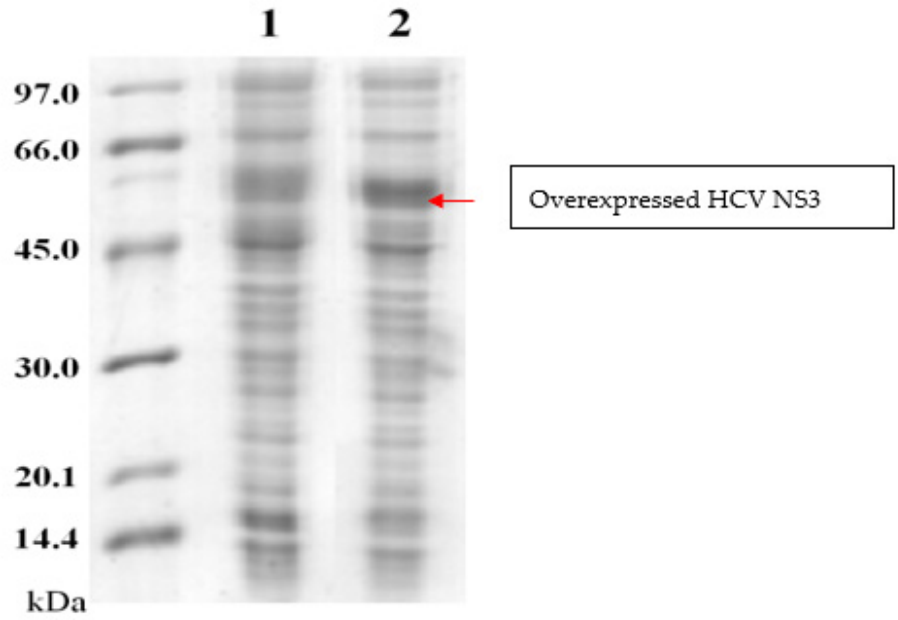
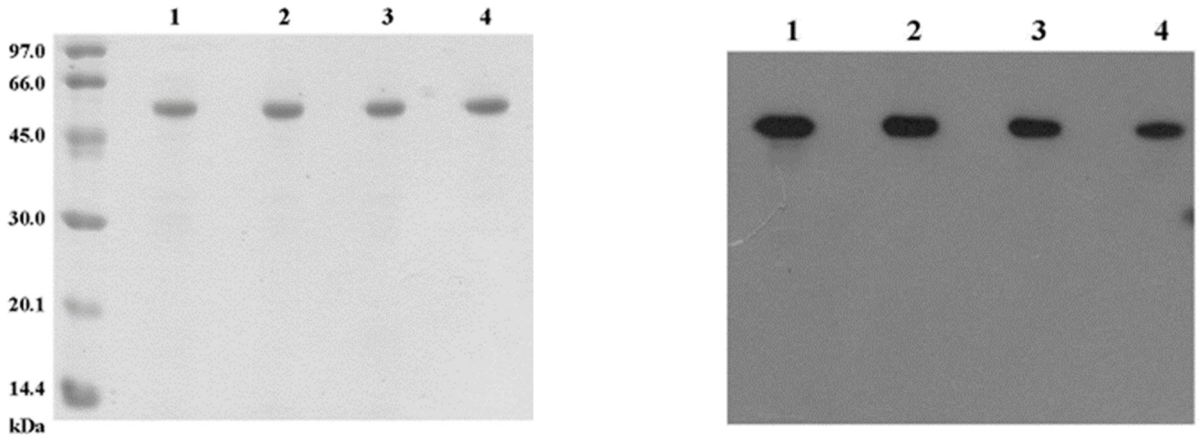


Figure 1. SDS-PAGE (12%) analysis of a crude extract. The supernatant fraction of cell lysate was incubated at 37 °C (lane 1) or 25 °C (lane 2). A low molecular weight protein marker was loaded and labeled their molecular weights.



(**a**) (**b**)

Figure 2. Purity and specificity of purified HCV NS3 antigens on SDS-PAGE (**a**) and western blotting (**b**). Lane 1 and lane 2 are long-term-stored and fresh NS3 antigens produced at 37 °C, respectively. Lane 3 and lane 4 are long-term stored and fresh NS3 antigens produced at 25 °C, respectively.

Table 1. Production yield of HCV NS3 in a 40 L reaction, with incubation temperatures of 37 °C and 25 °C.

Temperature	Crude Extract	HCV NS3	Production Yield
(°C)	(mg)	(mg)	(mg/L)
37	1030 (±5%)	166 (±3%)	4.15 (±3%)
25	910 (±4%)	445 (±1%)	11.1 (±1%)

2.1.2. Property and Stability of Fresh and Stored NS3 Proteins

The protein stability of NS3 after long-term storage was evaluated for their homogeneity and properties as compared to the freshly produced NS3 fractions in both proteins produced at 25 and 37 °C. After a single Talon affinity column purification, both fresh

NS3 recombinant proteins produced at an incubation temperature of 37 °C and 25 °C (Figure 2A: lane 2 and 4) showed a major band in their respective lanes of SDS-PAGE. Similar band intensity (without the smearing of degraded oligopeptides) also obtained from protein samples stored over six months (Figure 2A: lane 1 and 3) in a 10% glycerol solution at −20 °C. Their degree of purity was above 90% by HPLC (Figure 3). The purity obtained was 92%, 97%, 92%, and 97%, respectively, from fresh and stored samples from the 37 °C and 25 °C incubations. In these two experiments, there were no significant differences in antigenic efficacy between the fresh and the long-term-stored NS3 protein antigens. The SDS-PAGE was further analyzed for their specificities by western blotting, as shown in Figure 2B. Besides the major anti-HCV specified band, a smear fraction was found for the long-term-stored NS3 proteins produced at 37 °C (Figure 2B, lane 1). In contrast, no smear fraction (no protein degradation) was observed for the recombinant NS3 protein sample produced at 25 °C even after six months of storage. This finding illustrates that the protein stability of NS3 antigens possessed in these fractions depended on the applied incubation temperature for growth, although less on the storage time. Moreover, the degradation of long-term-stored NS3 incubated at 37 °C might be due to protease activity from the highly homogenous stock enzyme solution. Although the cloned HCV NS3 was not full-length, it still retains a part of the protease fragment region, therefore it still retains the protease hydrolysis properties. [18]. However, the recombinant protein NS3 degradation was still very low compared to other proteins reported [19,20]. Such property improves HCV resistance and for which two mutations have been reported: D168N and L153I [20]. Their molecular mechanisms are discussed in detail by recent studies covering the destabilization of receptor–ligand hydrogen bonds [18,20].

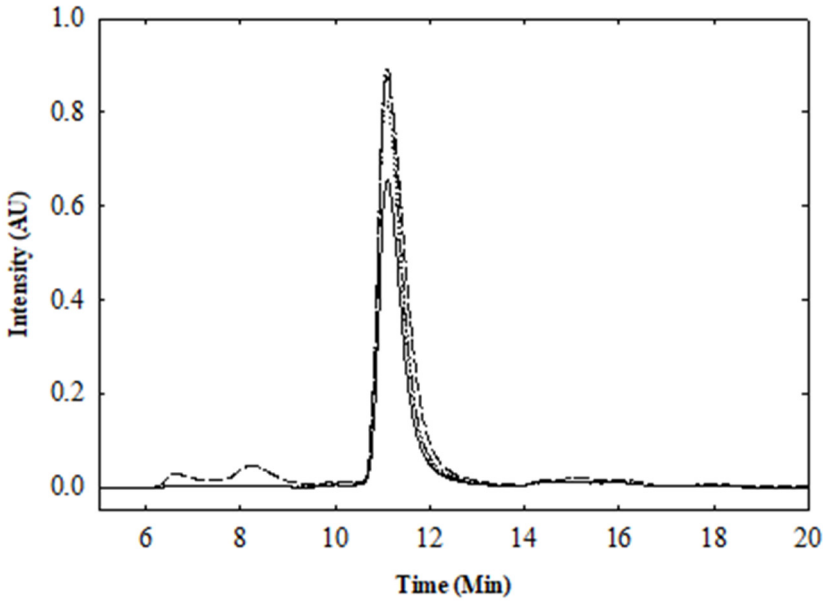


Figure 3. Size exclusion HPLC of purified HCV NS3. This assay was performed in a 0.1 M phosphate buffer at pH 6.7 by a flow rate of 0.3 mL/min using SEC column (BioSuite^{TM} 125, 4 µm HR SEC, 7.8 mm × 300 mm, Waters Corporation, Milford, MA, USA). All samples were eluted as a major 280 nm absorption peak (retention time near 12 min). The purified recombinant protein elution peaks of fresh 37 °C produced (-), fresh 25 °C produced (. . .), long-term-stored 37 °C produced (—), and long-term-stored 25 °C produced (-..), obtained in order.

2.1.3. Electrospray Ionization Mass Spectrometric (ESI-MS) Analysis

The deconvolutions of the mass spectra of fresh NS3 proteins produced at 25 °C or 37 °C gave the same molar mass of 54,540 atomic mass units. However, cluster peaks at around 1600–2100 m/z were detected only in 25 °C produced NS3 proteins (Figure 4A). When calculating the charge states of the monomeric NS3 (as the numbers labeled above

peaks in Figure 4), an additional peak in the NS3 produced at 25 °C was observed. These cluster peaks were isolated and deconvoluted as 109,080 amu belonging to the dimeric form of NS3. Even though the dimerization of NS3 was expected for its helicase activity [21], however, the monomeric form was still a major part of the 25 °C produced protein. The truncated recombinant HCV (for protease) is well-reported for its helicase activity [22,23]. The theoretical molecular mass of the above-mentioned protein was equal to 54,656.36 [24]; however, the obtained Mw from the mass spectrum was 54,540. In general, high m/z values signify either a higher molecular weight or a lower charge state for the protein. In most cases, unfolded proteins require more protons and would thus appear at lower m/z values than those of the folded forms [25]. Thus, based on these findings, it can be concluded that the NS3 protein produced at 25 °C was either more compact or with more dimeric form than those produced at 37 °C. According to the amino acid sequence, this protein has 15 cysteine units; thus, it can form a maximum of up to 6–7 disulfide bonds. Nevertheless, studies have not yet been carried out to confirm the number of disulfide bonds that are in place and their effects on its properties during the ELISA test. The disulfide bond is highly important for immunogenic and antigenic performance as well as in diagnosis. It was reported from mutagenic studies that the replacement of even a single cysteine out of six cysteine residues in herpes simplex virus type 1 glycoprotein resulted in either a great reduction or a complete loss of binding with those monoclonal antibodies recognizing irregular epitopes. However, there was no effect on its binding with monoclonal antibodies recognizing continuous epitopes [26].

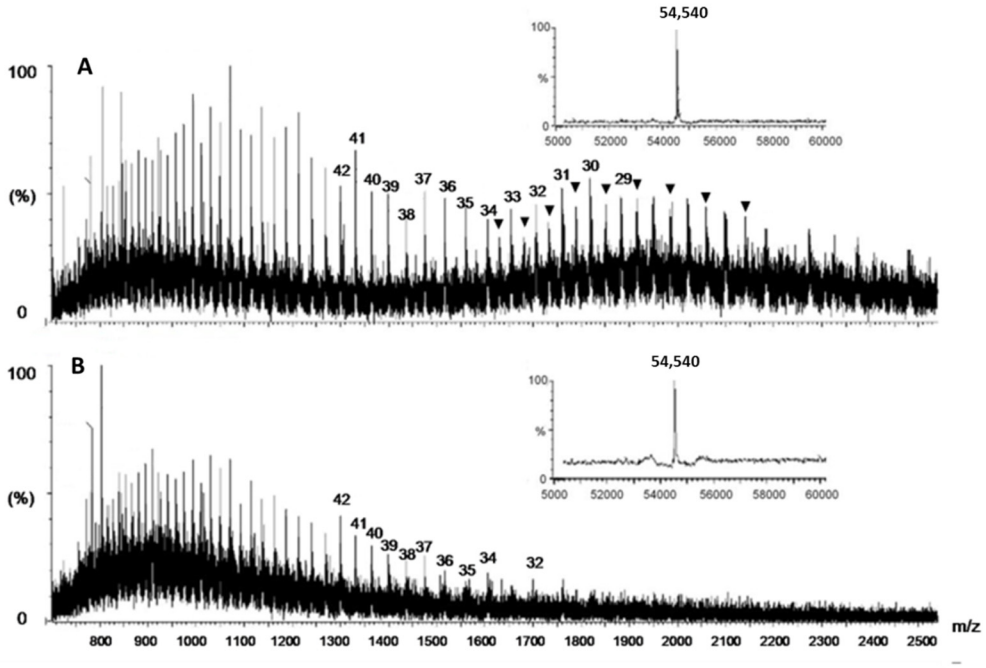


Figure 4. The mass spectra of HCV NS3 proteins produced at 25 °C (**A**) and 37 °C (**B**). They are plots of ion intensity vs. m/z (mass-to-charge ratio). The deconvolution of the spectra (inset) give the same molar mass of 54,540 atomic mass units. Numbers above the peaks stand for the z value (charge state) of the monomeric protein, and triangle labels are the signals specific to the dimeric protein.

2.1.4. Protein Structure Stability

The protein structure was analyzed by CD spectrometry. As shown in Figure 5, the far-UV CD spectra showing a negative peak at 222 nm and 208 nm were a CD spectrum typical for an α-helix conformation. However, at 210–220 nm, the elliptical values of long-term-stored NS3 proteins produced at 37 °C were significantly smaller than the other freshly prepared protein samples. It can be concluded that after long-term storage, the protein structure of the NS3 protein produced at 37 °C was slightly changed, followed by

aggregation and precipitation in the solution. The same phenomenon was described by a previous study carried out in 1999 [19]. This was also explained by the less compact 37 °C produced NS3 protein structure.

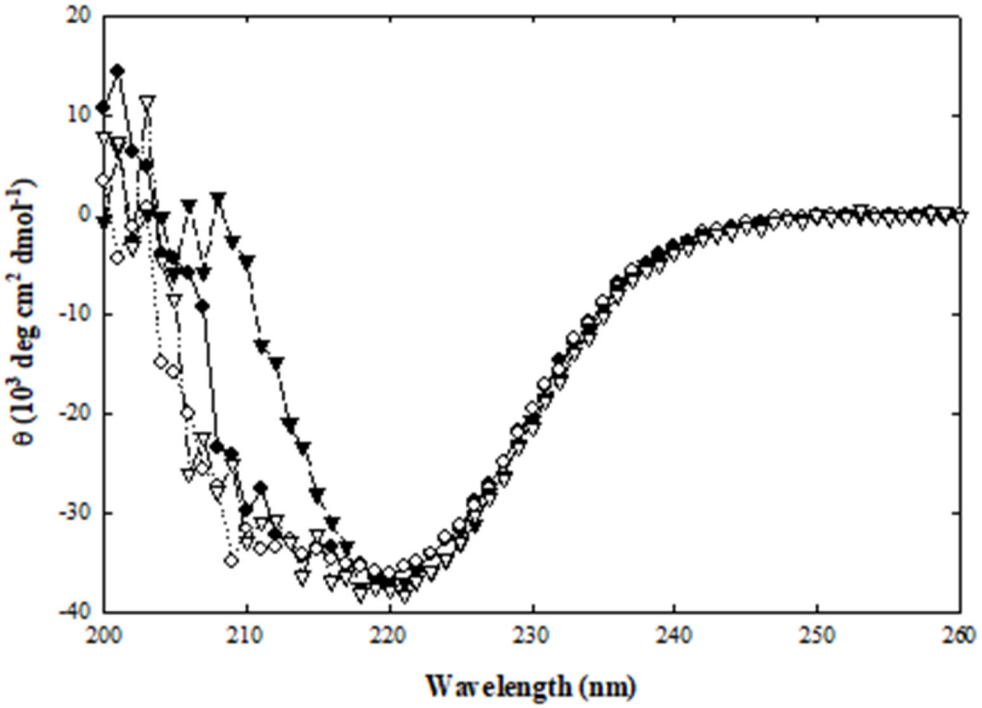


Figure 5. The far-UV CD spectroscopy of the HCV NS3 antigen recorded at 25 °C. These recombinant NS3 antigens include fresh 37 °C produced (•), fresh 25 °C produced (o), long-term-stored 37 °C produced (▼), and long-term-stored 25 °C produced (∇) protein samples.

2.1.5. Thermal Stability of NS3 Proteins

By monitoring the CD signal at 222 nm in a temperature range of 4–96 °C, the thermal denaturation of recombinant NS3 was measured. As shown in Figure 6, the CD values at 222 nm from 4 to 96 °C revealed that the thermal unfolding of all NS3 proteins began at around 40°C and was completed above 65 °C. When the unfolding fraction reached 50%, the observed temperatures were anticipated for the thermal denaturation (T_m) values at about 55–59 °C (as shown in Table 2). For a more quantitative evaluation of the temperature effect, the unfolding curves were analyzed with both the two-state and the three-state transition models. Even though the unfolding curves exhibited a typical biphasic transition profile, when the unfolding curve was applied to the two-state unfolding model, it failed to obtain the corresponding thermodynamic parameters (data not shown). Moreover, the theoretical curves of the three-state transition model were best fitted to the experimental data, as shown in Figure 6; the corresponding thermodynamic factors are summarized in Table 2. One possible explanation is that two domains are independently unfolded, and two transition phases correspond to the unfolding of individual domains. The whole unfolding process was subjected to the second transition phase in which Tm was almost equal to a 50% fraction unfolded. In the second transition phase, the loss of stability of long-term-stored NS3 induced at 37 °C was approximately 4.6 kcal/mol, a value that is explained by the disruption of several intramolecular interactions. However, advanced experiments are required to clarify this phenomenon. In general, these CD values shown in Figures 5 and 6 implied that all the recombinant NS3 possessed a similar structure and stability before the storage. After a six-month storage, however, the recombinant protein produced at a 37 °C incubation was less stable than that of the protein produced at a 25 °C incubation. There are three independent domains comprising this protein, including the helicase ATP-binding domain, the DEAD-like helicase C domain, and a domain containing

a 7α-helix (see Supplementary File). Moreover, the TI (T_m Index) of the DEAD-like helicase C domain was much higher than that produced by other proteins.

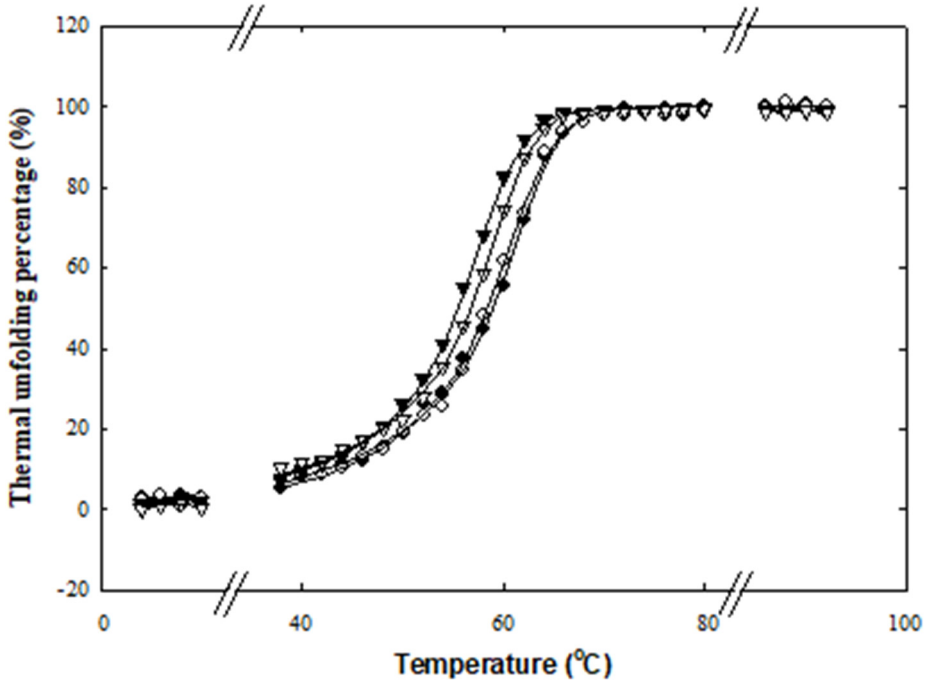


Figure 6. Thermal unfolding of HCV NS3 proteins recorded by far-UV CD spectroscopy. It was monitored by the change in ellipticity at a wavelength of 222 nm in the temperature range of 4–96 °C. The thermal denaturation (T_m) of fresh 37 °C produced (●), fresh 25 °C produced (o), long-term-stored 37 °C produced (▼), and long-term-stored 25 °C produced (∇) are obtained in order. Solid lines are the theoretical fitting curves with the thermodynamic parameters, also given in Table 2.

Table 2. Thermodynamic parameters for unfolding transition of NS3 Proteins produced at 37 °C and 25 °C.

	50% Unfolding	**First Transition Phase**					**Second Transition Phase**				
Sample [a]	T_m	T_m	ΔT_m	ΔH_m	ΔS_m	$\Delta\Delta G_m$	T_m	ΔT_m	ΔH_m	ΔS_m	$\Delta\Delta G_m$
	°C	°C	°C	kcal/mol	kcal/mol/K	kcal/mol	°C	°C	kcal/mol	kcal/mol/K	kcal/mol
F37	59	65.2	-	85	0.3	-	59.7	-	365	1.1	-
F25	59	64.0	−1.2	96	0.3	−0.3	59.1	−0.6	332	1.0	−0.7
L37	55	63.7	−1.5	81	0.	−0.4	55.5	−4.2	338	1.0	−4.6
L25	57	62.0	−3.2	89	0.3	−0.8	57.8	−1.9	387	1.2	−2.0

[a]: F37: Fresh 37 °C produced NS3; 25F: Fresh 25 °C produced NS3; 37L: Long-term-stored 37 °C produced NS3; 25L: Long-term-stored 25 °C produced NS3.

2.1.6. ELISA Specificity of NS3 Proteins

Two home-made HCV ELISA tests pre-coated by NS3 produced at 25 °C or 37 °C and two commercial kits, Abbott 2.0 and Ortho 3.0, were assessed by two BBI HCV panels (Boston Biomedica Inc., Easton, MA, USA), as shown in Figure 7A,B. In general, four ELISA tests demonstrated the same result: non-reactive specimens (S/Co < 1) contained negative samples and reactive specimens (S/Co > 1) contained positive and intermediate samples. It can be observed that the gap between the S/Co < 1 and the S/Co > 1 specimen presented on lane 4 was larger than the gap on lane 3. This indicated an improved specificity with 25 °C incubation. However, the specificity of NS3 was not higher than the commercial kits. It was noted that these two commercial kits were multi-antigen ELISA and our lab-made microplates were single-antigen ELISA, with only NS3 as the antigen. The antigenicity of truncated recombinant NS3 protein produced at the lab was comparable with the commercial one, while its production strategies were found easy and effective via

strategic low temperature expression. This method can be adopted in the near future to obtain potential products for HCV ELISA diagnosis.

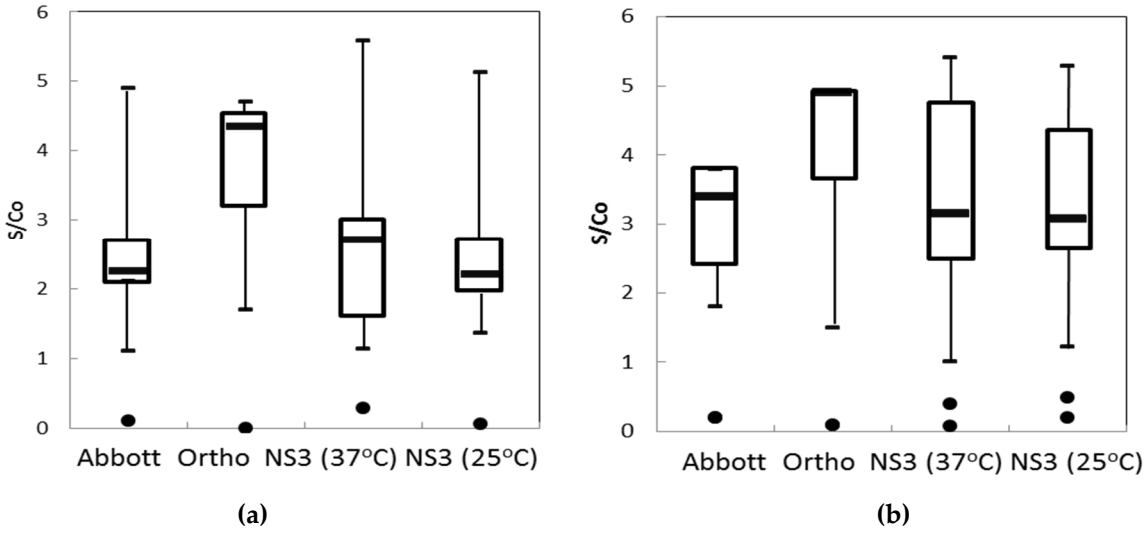


Figure 7. Box plots for ELISA specificity comparison of HCV NS3 antigens and two commercial kits. The experiments were performed using the Anti-HCV Low Titer Performance Panel PHV 105 (M) (**a**) and the Anti-HCV Mixed Titer Panel PHV 205 (**b**) as the standard specimens. The specimens of 7A include 7 positive samples, 1 negative sample (•), and 5 intermediate samples. The specimens of 7B include 20 positive samples, 2 negative sample (•), and 3 intermediate.

The study of Elleuche et al. [27] and Lawyer et al. [28] respectively discussed the truncated-form protein's benefits for specific activities over the full-length protein. The main reason for the truncated form of the HCV NS3 used in this study was to reduce its self-hydrolytic activity, which raises NS3 purity and reduces false negatives for the detection of anti-NS3 in ELISA (data not shown).

The expression at lower temperatures in an *E. coli* system helps to improve recombinant protein performance, which has been confirmed in the previous literature [29,30] and also confirmed in the results of our current study. Moreover, commercial biologic agents also mainly utilize lower temperatures for protein expression in an *E. coli* system, which illustrates this feature and advantage [31]. Additionally, the results of the current study are in line with the report of Sandomenico et al. [31], which confirms that the protein expression at a lower temperature (25 °C) exhibits superior performance as compared to that of the higher temperature (37 °C), as demonstrated by solubility, purity, antigenic efficacy, and stability.

3. Materials and Methods

3.1. Materials

All reagents used in the study were of analytical or molecular biology grade and purchased from Sigma or Merck. *E. coli* BL21 (DE3) (BF^- ompT *hsdS* ($r_B^- m_B^-$) dcm^+ *gal*λ (DE3)) purchased from Stratagene was used for cloning and recombinant protein expression experiments. Abbott HCV EIA 2.0 and Ortho HCV EIA 3.0 were purchased from Abbott and Ortho-Clinical Diagnostics, respectively. Two commercial HCV seroconversion panels for ELISA evaluation were purchased from BBI Diagnostics (BBI Diagnostics Boston Biomedica, Inc., Easton, MA, USA). These two BBI panels contained 37 specimens. Thirteen specimens of these species were obtained from Anti-HCV Low Titer Performance Panel PHV 105 (M). There were 7 positive, 1 negative, and 5 intermediate samples. The other 24 specimens were obtained from Anti-HCV Mixed Titer Performance Panel PHV 205. There were 20 positive, 2 negative, and 3 intermediate samples.

3.2. Expression of the Recombinant NS3 Proteins

A plasmid (pET 21a) carrying a gene code for a truncated NS3 flanked by a T7 tag and a His-tag was a gift from General Biologicals Corp. (GBC: General Biologicals. Corp., Taiwan). The expressed DNA of truncated NS3 was located from 3525 to 4971 bps on the HCV genome (the HCV NS3 helicase domain, amino acid residues 1175–1657, total of 483 aa) (GenBank ID: P29846) [32] and it was ligated into a pET-21a plasmid double-digested by *BamH*I and *Hind*III. The resulting plasmid was pET21a-NS3, which was well-characterized for its recombinant DNA sequence and protein sequence (see Supplementary File). The plasmid was expressed in BL21 (DE3) and the molecular mass of recombinant protein was determined. A single colony of BL21 (DE3) harboring HCV NS3 plasmid was used to inoculate in 400 mL LB (Difco) broth containing 100 μg/mL ampicillin, incubated overnight at 37 °C and then diluted to 40 L with LB-amp broth. After a 4 h incubation with shaking at 37 °C, or after bacterial density reached about 0.8 at 600 nm by spectrophotometer, IPTG was added as an inducer to a final concentration of 0.5 mM. Comparative studies of recombinant protein activity/efficacy were performed by an additional incubation of 4 h at 37 °C and/or 18 h at 25 °C.

3.3. Purification of the Recombinant NS3 Proteins

The harvested cells of BL21 (DE3) from the 40 L culture medium were suspended in 150 mL of Buffer A (50-mM Tris, 0.5 M-NaCl and 5-mM imidazole at pH 8.0) and lysed using a microfluidizer. The supernatant (crude extract) was separated by centrifuging (10,600× *g*, 60 min, 4 °C) and applied to Talon affinity column (BD Bioscience, 50 mL), which was pre-equilibrated with a 2-fold volume of Buffer A. After washing the unbound proteins with a 10-fold volume of Buffer A by gravity at 4 °C, the NS3 was eluted with 300 mL of the 100 mM imidazole (50 mM Tris, 0.5 M NaCl, and 100 mM imidazole at pH 8.0). Purified proteins were stored for further use in small aliquots (about 1 mL) at 4 °C within one week (fresh NS3) or at −20 °C over 6 months (long-term-stored NS3) for further experiments.

3.4. HPLC Performance

Identification was performed and purity of the recombinant proteins was analyzed using high-performance liquid chromatography (HPLC; 600E Multisolvent Delivery System, Waters Corporation, Milford, MA, USA) with an Ultra High-Resolution SEC column (BioSuite^{TM} 125, 4 μm HR SEC, 7.8 mm × 300 mm, Waters Corporation, Milford, MA, USA) under 4 °C. About 100 μL of purified recombinant proteins was applied and eluted with 0.15 M phosphate buffer (pH 6.8) at a flow rate of 0.3 mL/min [33]. The absorbance data at 280 nm (996 Photodiode Array Detector, Waters Corporation, Milford, MA, USA) were collected and processed using the Empower 2 Chromatography Data Software (Waters Corporation, Milford, MA, USA).

3.5. Western Blot Analysis and Protein Estimation

The purified recombinant NS3 was first analyzed by 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE). Its specificity and stability were then observed by western blotting using anti-His as the primary antibody [34]. The protein concentration and secondary structure were assayed by Bradford method (Bio-Rad, Hercules, CA, USA) and Circular Dichroism spectra (details in the section below), respectively.

3.6. Electrospray Ionization Mass Spectrometric (ESI-MS) Analysis

Mass spectra were recorded with a quadrupole time-of-flight mass spectrometer (Micromass, Manchester, UK). This was used to scan at a ratio of mass to charge in the range of 100–2500 units (m/z), with a scan of 3 s/step and an interscan duration of 0.1 s/step. In all the ESI-MS experiments, the quadrupole scan mode was used under a capillary needle at 3 kV, a source block temperature of 80 °C, and a desolvation temperature of 150 °C [35]. The desalted form of the NS3 proteins in 10% acetonitrile containing 0.1% formic acid used

for the MS measurements was normally within the range of 5–10 μg. Data acquisition and processing were performed with the MassLynx software (Version 4.0).

3.7. CD Measurements

The stabilities of secondary structure were performed by Circular Dichroism (CD) spectrometry. It was recorded using an Aviv model 202 Circular Dichroism Spectrometer equipped with a 450-watt Xenon arc lamp. Far UV CD data were expressed in terms of the mean residue ellipticity (θ_{MRE}) in deg cm^2 $dmol^{-1}$ using the following:

$$\theta_{MRE} = (100\ \theta_{obs}\ Mw)/(nlc) \quad (1)$$

where θ_{obs} is the observed ellipticity in degrees, MW is the protein molecular weight in g/mol, *n* is the number of amino acid residues, l is the path length of the cell in cm, c is the protein concentration in mg/mL and the factor of 100 originates from the conversion of the molar weight to mg/dmol unit. The concentration of the NS3 protein in CD measurements was about 0.1–0.3 mg/mL in the 1 cm cuvette.

3.8. Thermal Unfolding Experiments

Thermal unfolding curves of NS3 were experimentally obtained in a 20 mM phosphate buffer (pH 6.8) using an Aviv model 202 Circular Dichroism Spectrometer. The CD value at 222 nm was monitored while raising the temperature in the range of 4–96 °C, with 2 °C and 30 s intervals. The midpoint of thermal denaturation (T_m) was extracted from the thermal unfolding, curved by normalization CD value, as in the following equation:

$$X = (1 - Y_T/Z) \times 100\% \quad (2)$$

where X is the percentage of thermal unfolding protein, Y_T is the CD value at a given temperature T, and Z is the average CD value between 70 and 96 °C. The Tm was defined as the temperature when X = 50%.

The Tm and thermodynamic parameters of unfolding curves were analyzed by a three-state transition model. The enthalpy and entropy changes were calculated by the modified van 't Hoff equation [36]. The theoretical curve was fitted to the experimental data by non-linear least square fitting procedure to obtain the thermodynamic parameters for the individual transition phase. The difference in the free energy change of the unfolding between each NS3 and fresh 37 °C incubated NS3 was estimated at the Tm of fresh 37 °C incubated NS3 using the following formula:

$$\Delta G_m = \Delta T_m\ \Delta S_m \quad (3)$$

where ΔT_m is the difference in T_m between each NS3 and fresh NS3 incubated at 37 °C, and ΔS_m is the entropy change of fresh NS3 incubated at 37 °C at its T_m.

3.9. ELISA Analysis of the Recombinant NS3 Proteins

The purified recombinant NS3 diluted to 1.2 μg with the coating buffer (20 mM phosphate buffer, pH 6) was applied to microplates and the plates were then incubated at 4 °C for at least 20 h. The microplates were washed, overcoated, and dried for 20 h. Specimens in two seroconversion panels (BBI) were diluted 20-fold with the Specimen Diluent C (3HC03-350 Lot C58C06SDP, GBC). A total of 100 μL of the diluted mixture was then added to the microplates for 1 h at 37 °C as the primary antibody. After washing, the conjugated (100 μL, GBC) anti-human IgG-HRPO was then applied as the secondary antibody for 30 min at 37 °C. The microplates were washed and developed by adding 100 μL of 3,3′,5,5′-tetramethylbenzidine in the dark for 30 min at room temperature. The peroxidase reaction was terminated by adding 100 μL of 2-N H_2SO_4, and the absorbance at 450–650 nm was measured using the ELISA reader (Molecular Devices). The absorbance was compiled statistically as S/Co value (S: sample value; Co: cutoff value). The Co

value was calculated as the negative control OD value, plus the positive control OD value, divided by 4 (Co = NCx + PCx/4). Samples with an absorbance equal to or higher than the cut-off value, i.e., S/Co values greater than 1, were considered to be initially reactive in the assay.

4. Conclusions

This study confirms that the truncated form of the HCV NS3 protein as compared to the full-length protein exhibits better specific activity, purity, and application due to its reduced self-hydrolytic activity and false negative results in ELISA. Our results clearly show that the expression at lower temperatures in the *E. Coli* system improves recombinant protein performance. The incubation temperature shifting from 37 °C to 25 °C appeared to improve protein folding, solubility, the yield of the soluble fraction of HCV NS3 from 4.15 to 11.1 mgL^{-1}, and the storage shelf life of the cloned and over-expressed recombinant NS3. Such properties are probably due to a compact protein structure that increases the protein stability. When the specificity of NS3 expressed at 25 °C was compared with that of another NS3 and two commercial kits by international standard panels, the truncated NS3 produced at 25 °C had a better discriminating ability than the 37 °C produced protein, and was competitive with the commercial kit (Figure 7). Hence, it may have the highest potential for HCV ELISA diagnosis. These characteristics and advantages can be seen in commercial biologics, which mostly use *E. coli* systems for protein expression. Moreover, the results of the current study are in line with the published reports, which confirms that the protein expression at lower temperatures (25 °C) exhibits superior performance as compared to that at higher temperatures (37 °C), as demonstrated by solubility, purity, antigenic efficacy, and stability. In conclusion, a lower temperature expression technology of proteins offers greater potential for the development of biological agents and in vitro diagnostics that are both more effective and commercially feasible.

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