Overexpression of full-length core protein of hepatitis C virus by *Escherichia coli* cultivated in stirred tank fermentor

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Abstract

The mature core protein of the Hepatitis C virus (HCVC173) carrying pelB as a signal peptide (PelB::core) was overexpressed in *Escherichia coli* as 18% and 23.3% of the host's total protein, in flask and fermentor cultivation, respectively. A final specific yield of 25 ± 1 mg HCVC173/g dry cell weight and an overall productivity of 51±1 mg HCVC173/l/h were obtained in the stirred-tank fermentor. The recombinant PelB::core protein was overexpressed as the inclusion body (IB) form, higher than the expected level when compared to the HCVC173, which was also showed by the analysis of secondary structure of mRNAs and calculation of the Codon Adaptation Index of the gene. The results showed that the combined effects of protein fusion and the signal sequence significantly enhanced the production of recombinant mature HCVC173 in *E. coli*. Therefore, the fusion form of the mature HCV core protein and the conditions defined in this study provide an alternative strategy for HCVC173 production in high cell density culture of *E. coli*.

Keywords: Hepatitis C Virus; core protein; overexpression; recombinant protein

INTRODUCTION

Hepatitis C virus (HCV) has infected over 3% of people worldwide and is the major agent causing chronic liver infection, which often leads to liver cirrhosis and lethal hepatocellular carcinoma (HCC). Antiviral therapy against HCV has been shown to decrease the risk of HCC. The oncogenicity of several HCV proteins including structural (the core protein and the envelope glycoprotein E1 and E2) and non-structural proteins has been confirmed (Fung *et al*., 2009; Irshad and Dhar, 2006; Boulant *et al*., 2005; Feld and Hoofnagle 2005). Therefore, there is a high demand for the development of an HCV vaccine.

The HCV core protein is the most conserved structural protein that is derived from the initial 191 amino acids of a long open reading frame (ORF). It modulates the cellular processes, contributes to viral replication and pathogenesis and exhibits membrane protein features (Mohd-Ismail *et al*., 2009; Boulant *et al*. 2005; Mc Lauchlan *et al*., 2002; McLauchlan, 2000; Yasui *et al*., 1998). In fact, the recombinant HCV core protein can be considered as a prime vaccine candidate, a feasible vehicle or adjuvant for DNA vaccines, and a useful protein to study the epidemiology and biology of the HCV virus (Roohvand *et al*., 2007; Alvarez-Lajonchere *et al*., 2006; Irshad and Dhar 2006).

Different heterologous systems have been used to produce the recombinant HCV core protein in which either the truncated protein or its fusion forms carrying the His-tag and other proteins such as the maltose

binding protein (MBP) and glutathione S-transferase (GST) are expressed (Mikawa *et al.*, 2009; Hitomi *et al.*, 1995). Generally no- or low-level expression (Seong *et al.*, 1996; Songsivilai *et al.*, 1996) or formation of inclusion bodies (IB) (Boulant *et al.*, 2005; Linding *et al.*, 2003) have been regarded as bottlenecks in the production of the mature HCV core protein in *Escherichia coli*. Consequently many researchers have preferred to produce the truncated protein in *E. coli* or express it in other hosts (Martinez-Donato *et al.* 2006). Indeed, the truncated core protein is not folded properly and is not suitable for structural and conformational epitope studies. Moreover, the hydrophobic C-terminal region of the protein stabilizes the structure of the free mature HCV core protein (Acosta-Rivero *et al.*, 2005).

This study describes the cloning and expression of cDNA associated with the mature HCV core protein of an Iranian subtype (1a genotype, GenBank accession number: AF512996), coding for the first 173 amino acids of the HCV polyprotein (HCVC173), in the pET26b (+) based expression system carrying different leader sequences. For clarification of the experimental data, the primary structure and codon adaptation index (CAI) of the mRNA structures, and the amino acid content of the produced recombinant proteins were reassessed. Moreover, a possible strategy potentially useful for high expression of the mature HCV protein in a laboratory-scale fermentor was studied.

MATERIALS AND METHODS

Materials: Restriction enzymes and T4 DNA ligase were purchased from TaKaRa Co. (Japan). Protein marker for the determination of molecular mass, plasmid vectors and strains were obtained from Fermentas, Novagen and Stratagen companies (USA), respectively. Analytical-grade chemicals were obtained from Sigma and Merck (Germany).

Bacterial strains and growth conditions: *Escherichia coli* DH5α and BL21 (DE3) plysS strains were used as cloning and expression hosts, respectively. Luria-Bertani broth and agar supplemented with ampicillin (100 µg/ml) or kanamycin (30 µg/ml) were used for growth and isolation of the recombinant strains. LB medium or minimal medium containing KH_2PO_4 (19.95 g/l), $(NH_4)_2HPO_4$ (6 g/l), $MgSO_4.7H_2O$ (1.2 g/l) and trace elements solution (1 ml/l) were used for expression of the protein. The trace elements solution was composed of $FeSO_4.7H_2O$ (10 mg/l), $CaCl_2.2H_2O$ (2 mg/l), $ZnSO_4.4H_2O$ (0.5 mg/l), $SnSO_4$ $5H_2O$ (2 mg/l) and $Na_2B_4O_7.10H_2O$ (0.02 mg/l). The media supplemented with glucose, peptone or glycerol were used for the growth of bacteria and production of the recombinant protein in shake-flask culture. The semi-defined medium containing KH_2PO_4 (19.95 g/l), $(NH_4)_2HPO_4$ (3 g/l), $MgSO_4.7H_2O$ (2.5 g/l), trace elements (1 ml/l), supplemented with yeast (10 g/l) and glycerol (20 g/l) was used as preculture medium. Similarly, the same medium containing 30 g/l of glycerol was used for batch fermentation. Feeding during batch fermentation was carried out using a feed medium containing glycerol (750 g/l), $MgSo_4$ (15 g/l) and trace elements (1 ml/l). The $MgSo_4$ solution was sterilized separately.

Plasmid construction: Cloning experiments were performed according to standard methods described by

Table1. Plasmids used in this study.

Plasmids	Description	Reference
pBluscriptII KS (+) *Apr lacZ*	Cloning vector	Fermentas
pET-26b(+)	Expression vector	Novagen
pBluntcore	pBluscript II KS(+) with 519 bp mature HCV core cDNA	This study
pBluhiscore	pBluscript II KS(+) with 519 bp mature HCV core+ 21bp(Met + 6X his)	This study
pBlupelBcore	pBluscript II KS(+) with 519 bp mature HCV core cDNA	This study
pET- ntcore	pET-26b(+) with 519 bp mature HCV core cDNA	This study
pET-hiscore	pET-26b(+) with 519 bp mature HCV core + 21bp (Met + 6X his)	This study
pET-pelBcore	pET-26b(+) with 519 bp mature HCV core + pelB (66bp)	This study

Sambrook *et al*. (2001). Plasmids used in this study are listed in Table 1. Briefly, two 519 bp and 540 bp DNA fragments were amplified by PCR using coreF (5´-CACCATATGAGCACGAATCCTAAACC-3´, *Nde*I)/ coreR(5´CCAAGCTTCATTAAGAGCAACCAGGAAGG -3´, *Hind*III), or coreHisF (5´-GATATACATATGCAT-CATCATCATCATCACATGAGC-3´, *Nde*I)/ coreR primers. Similarly, another PCR was performed using primer pairs of coreMscF (5´-GCATGGCCATGAG-CACGAATCC-3´, *Msc*I)/ coreR to amplify a 519 bp DNA fragment. The PCR fragments were then cloned into the pBluescript II KS (+) cloning vector, leading to the construction of pBluntcore, pBluhiscore and pBlupelBcore plasmids. Subsequently, the fragments were excised from pBluntcore and pBluhiscore using *Nde*I/*Hind*III and from pBlupelBcore using the *Msc*I/*Hind*III restriction enzymes and then subcloned into the corresponding sites in pET26-b (+). The resulting recombinant plasmids were named, respectively: pET-ntcore, pET-hiscore and pET-pelBcore (containing pelB as signal peptide), and were subsequently transformed into *E. coli* BL21.

Protein expression studies
Flask-scale protein production: The LB medium (supplemented with 30 µg/ml of Kanamycin) inoculated with an overnight culture of recombinant cells was incubated at 37ºC until optical density at 600 nm (OD$_{600}$) reached 3.2. Then isopropyl l-beta-D-thio-galactopyranoside (IPTG) was added at a final concentration of 0.4 mM and incubation was continued for 2 h at 37ºC and then 3 h at 30ºC, with shaking at 150 rpm. The cells were harvested by centrifugation at 5000 g for 10 min. Total and soluble proteins were analyzed as described previously (Rastgar Jazii *et al*., 2007).
Protein production in fermentor: 1.8 liters of the semi-defined medium was inoculated with 200 ml of an overnight culture, and grown at 37ºC in a stirred-tank fermentor (BioFlo 3000, New Brunswick Science Corp. USA). The dissolved oxygen (DO) during the fermentation was controlled at 40% saturation by varying the agitation speed that ranged from 500 to 900 rpm, while the air flow rate was kept constant at 0.5 l/min. During the batch fermentation and feeding, the inlet air was enriched with pure oxygen when required.

When a suitable cell density (OD$_{600}$ = 15) was obtained at the early logarithmic phase of growth, the promoter was induced by the addition of IPTG at a final concentration of 0.4 mM and fermentation was then continued for 4 h at 37°C and another 4 h at 30°C. The pH was automatically maintained at 7 by HCl (20%) or NaoH (2 M). After a decrease in the growth rate during batch fermentation, exponential feeding was initiated using the feed medium. The volume of the feed was adjusted so as to reach a growth rate (µ) of approximately 0.3. After harvesting and lysis of the cells, the lysates were isolated and analyzed for HCVC173 expression. The optical density of the culture was measured as mentioned above and converted to the cell dry weight as described by Khalilzadeh *et al*. (2003).

In order to extract the insoluble recombinant protein, the cell pellet was resuspended in lysis buffer (50 mM Tris-HCl (pH 8.5), 10 mM EDTA, 100 mM NaCl, 2 mM DDT, 1 mM PMSF, 0.4% (v/v) Triton X-100 and 70 µg/ml of lysozyme) and exposed to three freezing (-70ºC)/thawing (37ºC) cycles. After the third thaw, DNase and MgSO$_4$ were added to final concentrations of 20 µg/ml and 20 mM, respectively. The suspension was then incubated at 37ºC for 30 min, sonicated 10 times for 45 s and 30 s intervals and then centrifuged at 18,000 g for 20 min. The resulting pellet was washed three times with washing buffer (50 mM Tris-HCl (pH 8.5), 10 mM EDTA, 100 mM NaCl, 2 mM DDT, 1 mM PMSF) and the IBs were then separated.

Gel electrophoresis and Western blotting: The expressed protein was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Western blotting was performed according to a previously described method (Hourioux *et al*., 2007). In the latter procedure, the polyclonal antibody from a commercial kit (Asia-Lion Biotechnology Co. China) and the rabbit anti-human IgG-horseradish peroxidase conjugate were used as primary and secondary antibodies, respectively.

N-terminal sequencing: N-terminal sequencing of the recombinant protein was performed according to the method of Edman using an ABI Procise 492 Edman Micro Sequencer connected online to an ABI 14ºC PTH Amino Acid Analyzer (USA) (Niall 1973).

***In silico* study of RNA and protein structure:** The Mfold (Zuker, 2003) (http://mfold.bioinfo.rpi.edu/) and GenScript's Optimum GeneTM softwares (http://www.genscript.com/cgi-bin/tools/rare_codon_analysis) were used to analyze
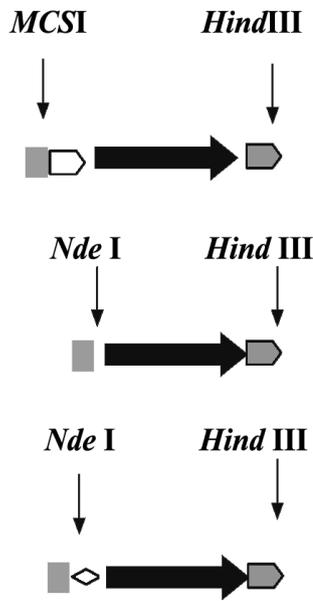
Figure 1. Shematic maps of three different cassettes corresponding to: *pelBcore*, *ntcore*, hiscore. Relevant restriction sites used for gene cloning with or without the pelB signal peptide are indicated. T7 promotor (■), PelB (▷), core gene (➡), His-tag 6x (◇) Stop codon (➡).

Figure 2. A: SDS-PAGE analysis of three recombinant HCV core proteins and B: SDS-PAGE and C: Western blot D: analysis of periplasmic fluid of the PelB::core expressing strain. M: Protein molecular weight SM0661 ladder, Fermentas, 1. Negative control (Plys), 2. pelBcore, 3. ntcore, 4. hiscore strain, 5. Total PelB::core protein, 6. Periplasmic proteins, 7. Cytosolic proteins, 8-10 Western blot corresponding to 5-7. 11,12 PelB::core IB and its souble form.

the secondary structure and codon usage of the HCV mRNAs, respectively. Regions covering the first 126 bp of the corresponding mRNA, flanking the start codon, were analyzed by the Mfold software. The potential disorder of proteins was predicted using the DisEMBL 1.5 software (http://dis.embl.de//). The signal peptide cleavage of the recombinant protein was predicted using SignalP 3.0 (Bendtsen *et al*., 2004).

RESULTS

Expression of recombinant mature HCV core protein: Three different recombinant plasmids, pET-ntcore, pET-pelBcore and pET-hiscore encoding, respectively, the whole mature HCV core protein (NT-core), the mature HCV core protein carring the pelB as signal peptide (PelB::core) and the HCV core protein with His (6x) tag (His::core) on its N-terminus were constructed (Fig. 1). The recombinant strains harboring the pET-ntcore, pET-pelBcore and pET-hiscore plasmids expressed the core protein with the expected 20 kDa molecular weight at different levels. Although all three strains expressed the core protein, the pET-pelBcore strain produced the most stable and highest level of expression (18% of the host's total protein) (Fig. 2).
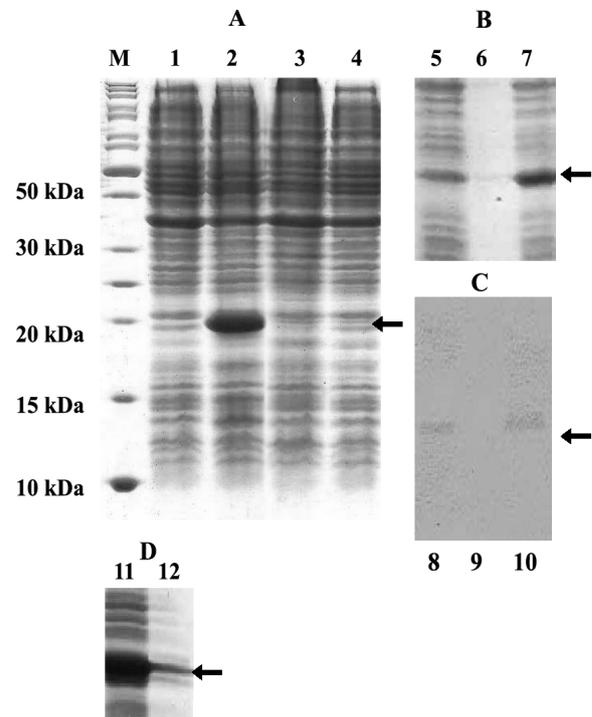
Periplasmic expression of core protein: Protein expression and secretion of PelB::core were evaluated in LB and minimal media supplemented with glucose, peptone or glycerol. Although the LB and the supplemented minimal media showed different expression levels, none of them could support the targeting of the soluble recombinant core protein into the periplasmic space of *E. coli* as demonstrated by SDS-PAGE and Western blot analyses (data not shown). Consequently, the minimal medium supplemented with glycerol was selected for high level production of the recombinant HCV core protein.

Induction conditions were optimized in order to improve PelB::core expression. However, no secretory target protein was detected. Protein analysis showed the expressed core protein mainly as IB and a small soluble fraction entrapped through the inner membrane that could be released during IB extraction by Triton X-100 treatment (Fig. 2D).

N-terminal sequencing of the overexpressed recombinant protein revealed that the pelB signal peptide was not cleaved from PelB::core protein, thus not allowing the translocation of the protein through the
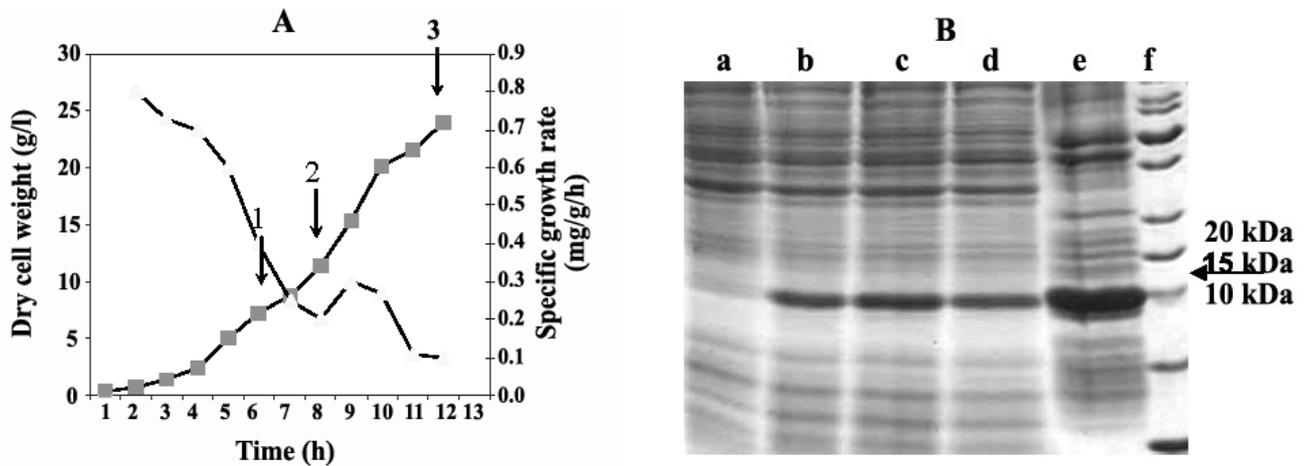
Figure 3. A: Growth kinetics of recombinant *E. coli* BL21 and B: PelB::core expression (20 kDa) during fed-batch fermentation at variable specific growth rates (0.10-0.8/h) in A bioreactor containing 2 l of the defined medium. Lane f: protein ladder (SM0661 Fermentas). A: 1, Induction time; 2, start of feeding and 3, harvesting time. B: a, negative control (before induction), b-e: PelB::core expression at 2, 3, 12 h after induction and 7 h after incubation of the final biomass at 25°C.

inner membrane. This and earlier observations raised important questions of why the PelB::core protein was overexpressed whereas the two other proteins were not, and why the signal peptide was not processed from PelB::core and whether overexpression of the PelB::core protein would continue during the scale-up process.

Analysis by the signalP software indicated that the pelB signal peptide could be processed and separated from the N-terminal of the PelB::core proetin in *E. coli*. However, this processing did not occur and prompted reassessing the probability of the effect of signal pelB on the aggregation of the fusion PelB::core protein. The probability of the potential disorders of PelB::core was analyzed and compared with its imma-ture (HCVC191) and mature (HCVC173) forms. These analyses indicated that the presence of the pelB signal creates a disorder segment (located in amino acids 10-17) in the PelB::core with over 50% disorder probability. This is 50% up when compared to HCVC173 but 50% down when compared to HCVC191 that shows a segment disorder (located in amino acids174-185) with 100% disorder probability. Therefore, compared to the mature protein, the PelB may have reduced the foldability of PelB::core and increased its aggregation to form IBs.

Protein poduction in fermentor: In order to examine the reproducibility of the large-scale production of protein required for downstream processing, a cultiva-tion procedure involving high-cell density culture (HCDC) in a stirred tank fermentor was designed. The preliminary experiments showed that the target protein was produced mainly during the exponential phase of growth. Therefore, the promoter was induced at the early logarithmic phase by adding 0.4 M IPTG. It was found that the pET-pelBcore strain had the highest level of expression, which reached a maximum 3 h after induction (Fig. 3B). In order to obtain large quan-tities of the recombinant protein, fermentation was

Table 2. Rare codon analysis of the recombinant constructs.

Recombinant Construct	CAI	Average GC content:	The percentage of low fre-quency expression
pelBcore	0.74	58.74%	8%.
hiscore	0.69	56.70%	12%
ntcore	0.64	64.06	16%.

Codon Adaptation Index (CAI), Average GC content and the percentage of low frequency expression of the genes (the first150 nt) were evaluated by GenScript's OptimumGeneTM softwares to analyze the secondary structure and codon usage of the HCV mRNAs.

allowed to continue by feeding the fermentor for 12 h, until an OD_{600} of 46 was achieved. A final dry cell weight (DCW) of 24 ± 0.1 g/l, together with the product yield and productivity of 25 ± 1 mg/g DCW and 51 ± 1 mg/l/h were obtained, respectively. Interestingly, maintaining the culture in a constant state at 25ºC for 7 h after the fermentation period, resulted in a 57% increase in the PelB:core levels (Fig. 2B).

DISCUSSION

In this study, three recombinant expression vectors carrying the cDNA for the HCV mature nuclear protein HCVC173 were constructed, two of which contained the PelB signal peptide or a His (6x) tag at their 5' ends. The recombinant vectors showed significantly different levels of expression under similar conditions. In fact, the PelB::core recombinant protein was expressed at an acceptable level constituting 18% of the host proteins, whereas the His::core and Nt-core had low rates of expression. While a maximum 4-5 mg/l of the mature HCVC protein has been previously reported (Hitomi *et al.*, 1995), 8-9 mg/l of the protein was produced in this study. Therefore, various parameters that affect the expression levels of recombinant proteins including mRNA stability, translation initiation, codon usage, fusion to other proteins, stability and protease sensitivity of the protein were examined in this study.

The impact of coding regions adjacent to the translation initiation codon, mRNA folding and rate of translation on expression levels in *E. coli* have also been reported (Bhattacharya *et al.*, 2005; Dethlefsen and Schmit, 2005; Kudla *et al.*, 2009; Sletta *et al.*, 2007). Comparison of different expression levels of the three mRNAs and differences in their initial regions imply that the codons for initiation of the pelBcore (pelB signal peptide) translation may have improved the expression efficiency of the recombinant PelB::core protein.

Since the efficiency of prokaryotic mRNA translation can be affected by primary and secondary structures within translation initiation regions (Moll *et al.*, 2002; zhang *et al.*, 2006), the three mRNA codons, mRNA structures and their CAI were re-examined to allow for interpretation of different expression levels. As a result, pelBcore showed a more reduced folding energy (ΔG) than the two other mRNAs, thus stabilizing mRNA by preventing its degradation. In a similar report, the crucial positive effects of the signal peptide

on the production levels of scFv-phOx, GM-CSF and interferon α-2b have been demonstrated in *E. coli* (Ramakrishnan *et al.*, 2010; Sletta *et al.*, 2007). In addition, the presence of the pelBcore initiation codon in the big loop may have enhanced the initiation of PelB::core translation.

In the pelBcore protein, 11.28% of all translated residues belong to the PelB signal peptide. This means that not only the initiation but also the rate of pelBcore translation may have been improved compared to the other two mRNAs. In the meantime, the increase in the pelBcore CAIs and reduction in the percentage of low frequency expression (or increase in the high frequency) (Table 2) have been resulted from its rare codon content caused by the PelB signal sequence.

In bacteria, the higher the translation activities, the more mRNA protection from degradation will occur due to ribosomoal occupation of the molecule, and this protection in turn will induce more mRNA stability and higher translation activities (Sletta *et al.*, 2007). It can therefore be concluded that pelBcore has higher rates of translation and thus, will enjoy higher rates of stability compared to the other two mRNAs, as shown in the case of scFv-phOx, GM-CSF. The higher than expected expression levels (twice as much as that in Nt-core) indicate that other determinants, such as its signal peptide may have assisted its expression levels to increase. Similar reports are available in the case of alkaline phosphatase and GM-CSF in *E. coli* (Le Calvez *et al.*, 1996).

The entire region of the HCV core protein has not been previously overexpressed in *E. coli*, and the GST-HCV core fusion protein has been expressed only when the core gene was truncated to the first 123 residues (Seong *et al.*, 1996; Songsivilai *et al.*, 1996). It has therefore been suggested that the hydrophobic C-terminal region of the core protein affects its expression. Although glutathione S-transferase (GST) and the maltose-binding protein (MBP) have been employed to enhance the solubility of their fusion partners, none of the hybrid HCV core proteins has been found to be soluble (Mikawa *et al.*, 2009; Hitomi *et al.*, 1995). Since the pelB signal peptide caused overexpression of the mature HCV173 core protein, it can be used as a preferred fusion partner over the GST or MBP for overeexpression of the mature HCV core protein and other similar proteins.

As the presence of the potentially disordered segments in the protein can decrease expression, foldability and stability of the expressed protein (Linding *et al.*, 2003), the probability of the potential disorders of

PelB::core was also analysed in this study. Indeed the signal peptide of pelB that has improved the expression level of PelB::core may have increased its aggregation due to containing a disorder segment with over 50% probability of beta aggregation. Therefore, when designing the hybrid protein, the presence of the potential disorder segment (s), at least the intrinsic disorder proteins such as the HCV core protein should be considered. On the other hand, the entrapment of a small soluble fraction in the inner membrane of the host could have been due to the nature of the membrane proteins and their interactions with the membrane (Boulant *et al*., 2005).

Since the HCV core protein is protease sensitive (Boulant *et al*., 2005), the hybrid PelB::core protein may decrease its proteolytic degradation and toxicity resulting in improved expression levels. A similar report on r-GM-CSF indicates that protein toxicity coupled with protease-based degradation is the principal reason behind its low productivity in *E. coli*. Moreover, dramatically increased expression levels of r-GM-CSF fused to His-tag have been reported (Bhattacharya *et al*., 2005). In this case, the expression levels can be improved by applying a larger fusion partner, such as MBP. Furthermore, it has been shown that the IB form does not necessarily inactivate the recombinant protein but in fact, the active IB can be used as an efficient catalyst for many bioprocesses (Garcia-Fruitos *et al*., 2007; de Groot and Ventura, 2006). Hence, the IB form of the HCV core protein may be useful for its handling and storage during downstream bioprocessing.

Most of the reported mature recombinant HCV core proteins are in the IB form, and there is also a report on soluble and low expression levels of HCV core120 and HCVcore176 in *Pichia pastoris* in which higher expression levels were achieved when the HCV core protein was expressed as a polyprotein together with either E1 or E1 and E2 proteins (Martinez-Donato *et al*., 2006). This report indicates that expression of the hybrid HCV core protein can be considered an efficient strategy for its production.

CONCLUSIONS

As a whole, the signal sequence effect and the fusion form of the PelB::core caused overexpression of the HCV core protein due to improvement of its mRNA stability, translation initation and higher translation activity. Moreover, the semi-defined medium and

strategy described and applied here in HCDC may be improved and used for production of the mature HCV core protein and other protease-sensitive and toxic proteins in *E. coli*.

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