

The effect of cell disruption techniques and chaotropic agents on the downstream purification process of mecasermin produced as inclusion body in *E. coli*

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Abstract

The isolation of the target protein from inclusion bodies (IBs) is a preliminary step to increase protein titer and to maintain its biological activity. In the present study, the effects of various cell lysis methods and the expression temperature was investigated on the improvement of the subsequent purification steps of mecasermin produced in IB. We also investigated the solubilization profile of the top-notch IB in 6 M guanidine hydrochloride (Gdn-HCl) and 8 M urea at different pH ranges. Mecasermin was expressed at various temperatures (25, 28, 30, and 37 °C) and the *Escherichia coli* cells were lysed by different cell lysis methods. The purity and quality of harvested IBs was evaluated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Finally, mecasermin was refolded and purified using gel filtration chromatography. The profile of SDS-PAGE analysis showed higher quality and purity after application of sonication coupled with lysozyme pretreatment for expressed mecasermin at 37 °C. Besides, from dithiothreitol application in washing step, we achieved a manifold enriched secondary IB for further purification of mecasermin. Mecasermin exhibited optimized solubility in 6 M Gdn-HCl at pH of 5.4. The findings of this study indicate an important role for cell disruption techniques to efficient purification of mecasermin. The study presents the most efficient techniques for improvement of downstream purification of mecasermin.

Keywords: Cell disruption; Inclusion body purification; Mecasermin; Strong chaotrope

INTRODUCTION

Insulin-like growth factor-1 (IGF-1) is a single chain polypeptide made up of 70 amino acids with three disulfide bonds (1). The major portion of IGF-1 in blood stream is in consistent with a ternary complex consisting of an acid-labile subunit (ALS), (IGFBP-3) and IGF-1 (2). ALS decreases IGF-1 transfer to extravascular area prolonging its half-life from minutes to hours and increases IGF-1 bioavailability (3). The mecasermin customized biosynthetic form of IGF-1 with molecular weight of 7.6 kD was synthesized by recombinant DNA techniques in 1986 (1). Mecasermin was proposed for short stature diseases and adjunctive pharmaceutical applications such as glucose homeostasis for diabetes mellitus and insulin resistance

conditions (1). The often-encountered outcome with high-yield expression of newcomer proteins within bacterial cell is the formation of accumulated intracellular particles termed as inclusion body (IB) (4). The fundamental gateway of recovering process for the intracellular proteins is cell lysis (4). Different methods have so far been utilized for cell disruption and targeted protein isolation. The chemical cell dispersion methods rely on highly specific interaction of a chemical element with cell wall structural units which permit proteins to passing along the cell barrier. The enzymatic cell breakage by additive enzymes such as lysozyme is widely employed for infraction of constitutive polysaccharides components of bacterial cell wall (5). Sonication is used for the emission of intermittent sonant waves with intense frequency

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to lyse bacterial cells and other. Also by means of the pressure-generating tools such as homogenizer cells could be disintegrated by pushing through a taper path (5). In the case of cysteine rich proteins, the possibility of IB formation is enhanced due to the inhibition of proper native disulfide connection under reductive media of bacterial cytosol (6). In the processing of these polypeptides, dithiothreitol (DTT) acts as a potent suppressor against accumulation of IB as an important challenge for purification. DTT is capable of preserving monothiol of the cysteines in reduced status (7). The recovered IBs are solubilized by the use of high concentrations of chaotropic denaturants such as urea or guanidine hydrochloride (4). Because of the complexity of protein purification from IB, finding a finely tuned and straightforward cell dispersion method can retain native-like structure of protein and scale up solubility and output of subsequent purification steps (8). Because mecasermin is used as a therapeutic drug, according to our previous work, we used the mecasermin gene in its vector without any tag (9-11). Thereupon, unlike tagged cases, mecasermin purification is very problematic via classic purification means such as affinity chromatography. In the current study, downstream purification processes are based on isolation of protein of interest as IB from other host proteins and impurities. The purpose of the present study was to explore different cell lysis methods for shearing the cell wall of Origami B strain of *Escherichia coli* (*E. coli*) and also to assess the expression temperatures for improvement and simplification of further mecasermin purification steps such as solubilization and refolding. This is the first time that the effect of different cell disruption methods is investigated to improve the downstream purification process of mecasermin expressed as IB. Finally pure mecasermin with higher solubility and monomeric native folding was obtained.

MATERIALS AND METHODS

Bacterial strain (*E. coli* Origami B) was provided by pasture institute (Iran). Ampicillin supplied by Sigma (USA). Isopropyl-beta-D-thiogalactopyranoside (IPTG) and protein

marker ladder were obtained from Thermo Scientific™ (Germany). Lysozyme was purchased from Cinnagen (Iran). Triton X-114 purchased from Sigma Aldrich (USA). The DNase and RNase enzymes were supplied by Sinaclon (Iran). The poly vinylidene fluoride (PVDF) membrane was provided by Amersham (UK). The primary and secondary antibody for IGF-1, diaminobenzidin and western blotting multicolor ladder were purchased from Santa Cruz (USA). Bradford assay reagent was provided by (Bio-Rad Laboratories GmbH (Germany). All other chemicals were from Merck (Germany).

Mecasermin production under various temperatures

Initially, expression of mecasermin was carried out via standard conditions as we reported in our previous studies (9,10). Expression of mecasermin was conducted over a narrow range of induction temperatures including 25, 28, and 30 °C. It is noteworthy that the conditions of expression such as the IPTG concentration, the culture compositions and expression host have been optimized in our previous works (10,11).

Bacterial cell disruption by high pressure homogenization

Initially, (1 g) of the thawed *E. coli* wet cells paste resulted from mecasermin expression at 37 °C, resuspended in 20 ml of 50 mM potassium phosphate buffer (17 mM KH₂PO₄ and 72 mM K₂HPO₄, pH = 7.4). The suspension was centrifuged at 6000 g for 30 min at 4 °C (Sigma 3k30, R 12158 Germany). This step was repeated twice. After centrifugal separation, the isolated pellet was resuspended in 20 ml of lysing buffer A (50 mM sodium acetate, 1 mM PMSF, 10 mM MgCl₂, 100 mM NaCl, 5 mM DTT, pH = 5.4) and mixed thoroughly with vortex (IKA MS 3 digital, Germany). The crude suspension was broken with homogenizer (NIRO SOAVI, Italy) at 1200 bar 4 times at 4 °C. We repeated this step for all of the wet cell pastes resulted from various induction temperatures (25, 28, and 30 °C). The pH of lysis buffer, the pressure and the times of homogenization and the concentration of the salts and DTT were optimized in this work.

Lysozyme pretreatment coupled with ultrasonication

In this technique washing was accomplished as mentioned in previous section. The isolated pellet was resuspended in 20 ml of lysing buffer A and mixed using vortex. This lysate was pretreated with 0.025 mg/ml lysozyme and incubated for 30 min at RT. The suspension was sonicated for 7 cycles of 5 s pulse with 2 min intervals at 25% of the maximal acoustic power using a sonicator (Bandelen Sonoplus, Germany) on ice. We repeated this step for all of the wet cell pastes resulted from aforesaid induction temperatures. The time and the number of sonication cycles, maximal acoustic power of sonicator, concentration of lysozyme and incubation time were optimized.

Mechanical cell lysis via ultrasonication process

In this method, washing of isolated IB was performed as previously described. Afterwards, the isolated pellet was resuspended in 20 ml of lysing buffer A. The resulting suspension was then disrupted by sonication for 15 cycles of 5 s pulse with 2 min intervals at 25% of the maximal power. We repeated this step for all wet cell pastes resulted from aforesaid cultivation temperatures. The time and the cycle number of sonication and maximal acoustic power of sonicator were optimized.

Enzymatic lysis using lysozyme treatment

In this approach, washing was performed as previously explained. Afterwards, the isolated pellet was resuspended in 20 ml of lysing buffer A and mixed using a vortex. The lysozyme was added to the lysate at concentration of 0.5 mg/ml. The suspension was shaken and incubated for 60 min at RT. This step was applied for all wet cell pastes resulted from expressed mecasermin at various temperatures. We optimized the concentration of lysozyme and the incubation time for this experiment.

Elevated recovery of IB by sequential washing of lysates

In order to remove impurities, all post-lysis mixtures were washed with lysis buffer B (1 M urea in buffer A pH = 5.4) as stated in our previous work (10,11).

Effect of DTT on the aggregation behavior of mecasermin polypeptide

One gram of wet cell paste resulted from expressed mecasermin at 37 °C resuspended in 20 ml of potassium phosphate buffer and washing was accomplished using lysis buffer A without DTT. The lysate was pretreated with lysozyme and sonicated for 7 cycles of 5 s pulse with 2 min intervals at 25% of the maximal power. This washing step was carried out in buffer B without DTT (10). Resulting fractions were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting analysis. The concentration of the DTT was optimized.

Evaluation of effectiveness of expression temperatures and cell disruption methods by SDS-PAGE analysis

All of the isolated washed fractions including supernatants and pellets from previous steps were subjected to SDS-PAGE. After electrophoretic separation the averages of bands intensity were calculated using Quality One software Version 4.6.3 (Bio Rad)

Verification of isolated IB by western blotting technique

All samples of supernatants and pellets resulted from previous steps were loaded into the wells of 17.5% gel. After fulfillment of SDS-PAGE, the SDS-PAGE was electro blotted onto a PVDF membrane and probed with the antibodies against IGF-1. The membrane was masked with 10 ml BSA (3 w/v %) in TBS-T solution for 1 h at room temperature on shaker to prevent nonspecific antibody reactions with membrane. Following 75-min incubation with primary rabbit polyclonal IGF-1 antibody at RT on shaker at a dilution of 1:500 in TBS-T and washing with TBS-T solution, the horse radish peroxidase (HRP)-conjugated goat anti rabbit-IGF-1 as secondary antibody was added at a dilution of 1:10000 in TBS-T solution. Afterwards, the PVDF membrane was immersed in diaminobenzidine solution containing H₂O₂ for 5 min (0.5 mg/ml DAB, 0.1% H₂O₂) and the signals on membrane were investigated.

Solubility profile and refolding studies of IB expressed mecasermin

At the outset, 100 mg of the recovered top-notched IB was resuspended in 3 separated falcons containing 4 ml of denaturant solutions (25 mg/ml) consisting of (50 mM Na-Acetate buffer, 100 mM GSH, 1 mM EDTA plus 6 M Gdn-HCl) with different pHs (4, 5.4 and 6.4) at RT. Also the same amount of recovered IB was dissolved in 30 mM Tris buffer with the same listed substances in Na-acetate buffer and pH was adjusted for each at pH (7, 8.1, 9). Similarly, for the pH range of (10-12), we continued solubilization with 50 mM glycine/NaOH buffer. After gentle vortexing, the process was continued at 22 °C. All of the samples were centrifuged at 32397 g at RT. The absorbance of the suspension was read at 280 nm using (CECIL. UV/VIS spectrophotometers, USA) three times and the average of solubilized protein was calculated ($1A_{280} = 1.71$ mg/ml of mecasermin). In the same way as mentioned above we repeated solubilization in 4 ml of (50 mM Na acetate buffer, 100 mM GSH, 1 mM EDTA plus 8 M urea). We centrifuged the soluble phases at 4 °C at 32397 g. After solubilization of the isolated IB we subjected all of the soluble and insoluble fractions to SDS-PAGE 17.5% for further investigation. Then, 4 ml of solubilized IB in (6M Gdn-HCl pH 5.4) buffer was refolded as mentioned in our previous work (10). Fourier transform infra-red (FTIR) and mass spectroscopy were conducted to confirm the folding of mecasermin.

Further refinement of the mecasermin via gel filtration chromatography

Refolded mecasermin was purified via gel filtration chromatography as mentioned in our previous work (10). The resulted fractions were pooled and the analysis

of protein fractions was performed on 17.5% SDS-PAGE.

RESULTS

Determination of optimum cell disruption technique and expression temperature

In the first step, we investigated the effect of various induction temperatures on the recovery of IB with high purity and minimum protein waste during the washing steps. Table 1 represents the percent of mecasermin between soluble and insoluble fractions resulted from different cell lysis methods performed at different induction temperatures subjected to 17.5% gel SDS-PAGE. Evidently at lower temperatures than 37 °C (25, 28, and 30 °C) in all four lysis methods tested a significant amount of the mecasermin was washed off and wasted. At 37 °C, the higher levels of target protein remained in the form of insoluble IB as indicated by higher percentage of IB form of mecasermin. Although proper protein folding was attained at lower temperatures but with regard to our purification method, 37 °C was the optimum temperature for expression of the mecasermin. The intensities of produced mecasermin as IB form at 37 °C for homogenization, simultaneous use of lysozyme and sonication, sonication and lysozyme pretreatment methods were 83%, 92.1%, 99.9% and 59.1%, respectively.

The SDS-PAGE panel in Fig. 1 shows the lysozyme pretreatment coupled with sonication. From comparison between the SDS-PAGEs (not shown for all gels) it was found that the concomitant use of 0.025 mg/ml lysozyme and sonication with 5 s on-off for 7 cycles at 25% of the maximal amplitude had the best effect on the quality of isolated IB and resulted in the maximum release of more pure product at 37 °C (Fig. 1).

Table 1. The ratios of (%) of mecasermin distribution between IB and soluble phases resulted from different tested cell disruption methods and cultivation process on 17.5% gel.

Cell disruption method	Expression temperature of mecasermin			
	37 °C IB/soluble	30 °C IB/soluble	28 °C IB/soluble	25 °C IB/soluble
Homogenization	83/17	76/24	76/24	72/18
Sonication and Lysozyme	92.1/7.9	69/31)	65/35	58/42
Sonication	99.9/0.1	75.6/24.4	69.3-30.7	60.9/39.1
Lysozyme	59.1/40.9	53/47	49.2/50.8)	46.3/53.7

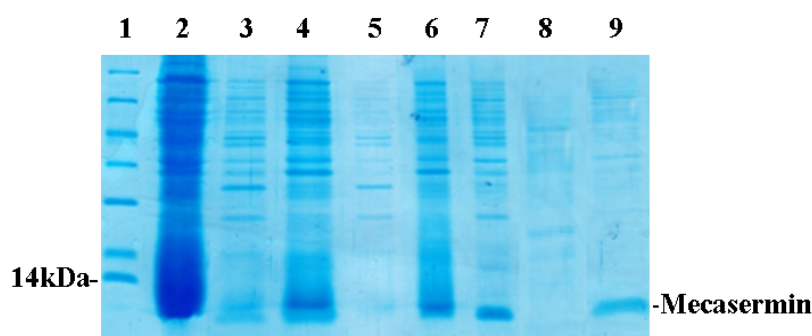


Fig. 1. The SDS-PAGE pattern of optimum cell lysis procedure (lysozyme pretreatment plus sonication) for IB formed mecasermin on 17.5% acrylamide gel. Different lanes contain the following: Lane 1; molecular weight marker (kDa), Lane 2; the lysate after sonication with lysozyme treatment in lysis buffer A, lane 3; first resulted light phase from washing with lysis buffer A, lane 4; first resulted sediment from washing with lysis buffer A, lane 5; the supernatant of second washing with lysis buffer A, lane 6; resulted sediment from secondary wash with buffer A, lane 7; resulted sediment from washing with buffer B (1 M urea + buffer A), lane 8; the supernatant of washing with buffer B, lane 9; final obtained washed pellet from washing with triton x-114 in buffer B. The position of mecasermin is indicated by the arrow.

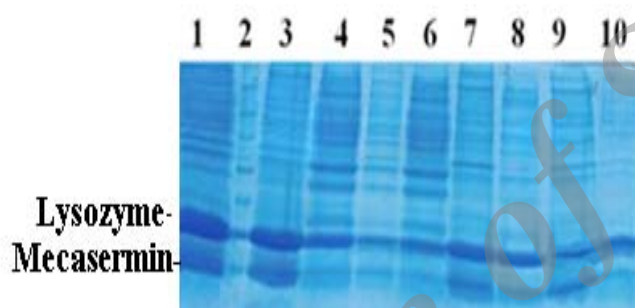


Fig. 2. Documentation of SDS-PAGE analysis for mecasermin distributed between pellets and supernatant fractions after bacterial cell lysis by lysozyme treatment on 17.5% gel. Different lanes contain the followings. Lane 1; the lysate after lysozyme treatment plus RNase and DNase. Lane 2; molecular weight marker (kDa), lane 3; the first pellet after washing with buffer A, lane 4; the light phase from first washing with buffer A, lane 5; resulted sediment from second washing with buffer A, lane 6; the supernatant of second wash with lysis buffer A, lane 7; resulted pellet from washing with buffer B (1m urea + buffer A); lane 8; the supernatant of first washing with lysis buffer B, lane 9; the resulted pellet obtained from second washing with buffer B along with triton x-114, Lane 10; the final resulted supernatant of final washing with triton x-114 in buffer B. The position of IGF-I and lysozyme is indicated by the arrows.

The mecasermin release and its purity and IB mass profile with homogenizer is closely similar to that of simultaneous use of lysozyme and sonication. Therefore, the aforesaid technique is more appropriate and effective method for isolation of mecasermin from IB compared to lysozyme and sonication techniques. With high sonication intensity, the host cells were completely disrupted which consequently resulted in greater amount of impurities. Fig. 2 represents the effectiveness of cell lysis by the use of enzymatic digestion of cell wall of *E. coli* Origami B via lysozyme. The intensity of the bands corresponding to mecasermin in the lysozyme treatment was dramatically reduced on SDS-PAGE.

Lysozyme by itself is not an efficient method for lysis of the cell wall of origami B *E. coli*. Using an appropriate temperature for expression, promising cell lysis method and an efficient washing process, more than 92% of IB containing mecasermin was isolated from *E. coli* cells. The mecasermin quantity and quality obtained using sonication and lysozyme and homogenization was much superior to the other lysis techniques examined in the current study. Up to this end, the top-notch IB isolated from production of mecasermin at 37 °C in the host cells fragmented by lysozyme pretreatment followed by sonication was adopted for further purification.

Solubilization and refolding of isolated secondary IB

Table 2 represents the IB solubilization of the adopted top-notch secondary IB in 6 M Gdn-HCl and 8 M urea at pH ranges of 4-12. Mecasermin exhibited the highest solubility in 8 M urea at pH 11 and 12. High solubility of mecasermin was also observed between pH 4 to 12 ranging from 12.5-22.25 mg/ml in 6 M Gdn-HCl. To achieve the optimum yield, refolding was performed at 5.4 in which mecasermin had a high solubility (75%) and chemical stability.

In general, the pH of final protein purification conditions such as refolding was kept around 5.4 near to the stable drug formulation pH value (50 mM Na acetate, 100 mM NaCl pH 5.4). During the refolding process the resulting solution was very clear and no precipitate was observed. FTIR spectrum inspection of refolded mecasermin revealed a band at 1655 cm^{-1} and a molecular

mass of 7.6 kDa for purified mecasermin in H NMR spectroscopy. The summarized data from solubilization have been presented in Table 2.

DTT suppresses the higher aggregation of mecasermin chains

Western blotting profiles (Fig. 3) indicated that DTT minimizes mecasermin aggregation resulting from intra- and inter-molecular disulfide linkages between cysteine residues of mecasermin. These panels proved that DTT can be used as reductive agent for sulfhydryl groups of mecasermin and can enhance refolding process. In the absence of DTT, the salient bands at 15 kDa and 24 kDa shown in Fig. 3B are indicative of stabilized nonnative disulfide bonds between cysteine residues under non-reducing condition in washing buffer. With DTT we obtained the best recovered IB comprising of monomeric mecasermin with high quality to carry on further purifications.

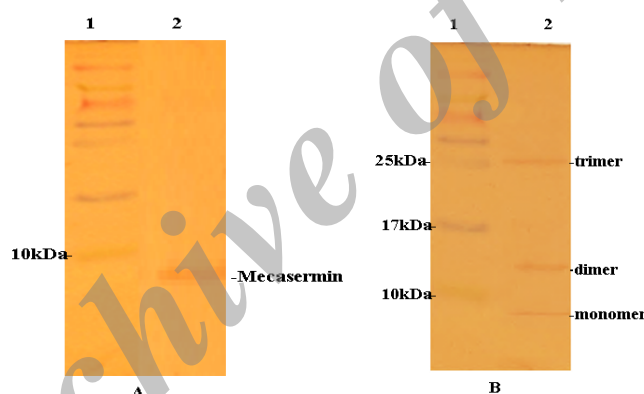


Fig. 3. Western blotting panel for final washed pellet from washing phase A; in the presence of 5 mM DTT or B; absence of DTT. In A: lane 1 contains western blotting multicolor ladder and lane 2 contains the final pellet of washing process with buffer B containing DTT. In B: lane 1 contains western blotting multicolor ladder and lane 2 contains the final washed pellet of washing process with buffer B without DTT. In the B the bands at positions of 15 kDa and 24 kDa, are indicative of the presence of IGF-I antibody against mecasermin polypeptides aggregates as ligands.

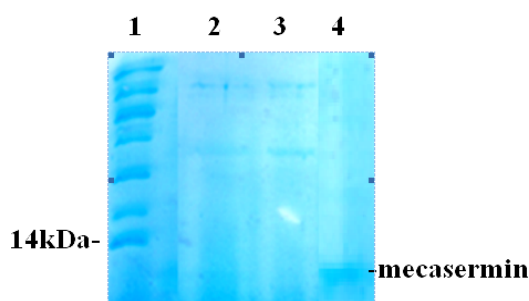


Fig. 4. The SDS-PAGE analysis of eluted fractions of the gel filtration column with Superdex beads. Lane 1; molecular weight marker. Lane 2, 3; eluted fractions containing protein impurities with molecular weight greater than mecasermin. Lane 4; eluted fractions containing pure mecasermin.

Table 2. Mecasermin solubility in different chaotropic agents.

pH	8 M Urea (mg/ml)*	6 M Gdn-HCl (mg/ml)*
4	3.5	17.5
5.4	2.5	18.75
6.4	1.75	18
7	1.25	13.25
8.1	4.5	12.5
9	8	20.5
10	15	20
11	22.5	21.25
12	23	22.25

Final purification of mecasermin by gel filtration chromatography

Fig. 4 represents the electrophoretic panel of eluted fractions from the column with Superdex beads. The prominent single band at a molecular weight of 7.6 kDa shows the monomeric mecasermin with maximum purity and was found to be homogeneous. This result represents that other impurities have been removed through gel filtration.

DISCUSSION

The method for cell disruption as a key factor to extract IB formed target proteins from host cells affects the downstream purification process of product such as solubilization and refolding (12). In our previous study we have optimized the expression conditions of mecasermin (9,10). To probe the effect of various growth temperatures, we expressed mecasermin at given temperatures under standard conditions (9,10). High temperature (37 °C) is appropriate for higher yield of mecasermin expression (10). In this regard, our result is similar to the work of Huang and coworkers (13). The IBs formed at lower temperatures exhibited a higher solubility in the first washing step. But according to our purification method a great deal of protein was lost in the supernatants following cell detachment and subsequent washing steps (14). Chisti and coworker have reported that the design of a cell disintegration procedure is an integral part of the downstream purification steps such as solubilization and refolding (14). The constraint of the disruption affects the physical characteristics of the lysate such as viscosity, compactness, size of the particles and

navigation of sedimentation, which influence the subsequent purification steps (15). Menzella and coworkers recovered the IB formed Prochymosin from *E. coli* cell via sonication along with lysozyme treatment and refolded this protein efficiently and inexpensively (16). Cherl-Ho and colleagues demonstrated different mechanical and chemical treatments effect on the extractability, density, sedimentation property, particle size and viscosity of the *Candida* cells. Their results showed that this morphological changes with different treatments affected subsequent processing and the extractability of proteins in the cell suspensions (17). Peternel and coworkers compared the efficacy of the enzymatic lysis via lysozyme and high-pressure homogenization and sonication to isolate of granulocyte colony stimulating factor (G-CSF) from bacterial cells (15). Their results showed that lysozyme attaches to the surface of the IBs, and it could not be removed by simple washing. This incident resulted to additional impurities in isolated IB. They concluded that enzymatic lysis by itself is not suitable method for IB isolation (15). Also, they demonstrated that the enhancement of ultrasonic power can increase damages of protein structure and increase impurities (15). In the present study protein impurities in lysozyme digestion was higher than that of the lysozyme pretreatment coupled with sonication because of longer incubation time and higher amount of lysozyme needed for the cell disruption.

High-pressure homogenization also caused some damage to the IBs, however the protein impurities was negligible (15). Anand and colleagues employed the enzymatic pretreatment along with mechanical cell lysis

to improve the efficacy of cell lysis and facilitation of the subsequent purification process by scaling up the amount of IB purity (18). In the present study we evaluated the effect of these methods in addition to sonication and lysozyme to improve downstream purification steps of mecasermin. Principally, high accessibility of IB surfaces via surrounding water molecules assists to the further solubility and refolding yield (19). In the present study the solubility of recovered top-notch IB was evaluated in different chaotropic denaturants such as urea and Gdn-HCl at various pH ranges. The mecasermin solubility in Gdn-HCl was more than urea. Our results about the effect of these denaturants are similar to the results of the study of Chandra and coworkers (20). In the urea buffer the precipitation was observed at initial pHs due to the suboptimal urea function in solubilization of the IB. However, the higher pH exerts irreversible chemical modifications on mecasermin and leads to lesser activity. Therefore, solubilization at higher pH may not be applicable for proteins with therapeutic grade. Hence, we selected the solubilized fraction in 6 M Gdn-HCl at the pH of 5.4 for further refolding process considering that in an acidic media (the pH lower than 3 the glutamine and asparagine amino acids are subjected to deamination). Therewith, at the pHs above 7, the possibility of methionine, cysteine and tyrosine oxidation in the mecasermin chain increases and this leads to protein deconformation. Furthermore, at the pHs close to isoelectric point (pI mecasermin 7.61) the protein solubility is very low (21). Our results indicated that alkaline pH (pH>8) can enhance the solubility of mecasermin however decreases the active yield of the product. Patra and colleagues isolated growth hormone with high solubility from *E. coli* at pH of 12.5 (22). Although pHs higher than 12 have been used for solubilization of proinsulin and growth hormones but these pHs can lead to lesser activity of the target product (22,23). We determined the effect of DTT to inhibit the higher aggregation of mecasermin chains as one of the parameters for improvement of the solubilization and refolding yield (7). The results showed a higher aggregation of

mecasermin chains in the washed IB in the absence of DTT (Fig. 3). We refolded mecasermin to optimum structure to progress final gel filtration purification step. FTIR spectrum of refolded mecasermin indicated a band at 1655 cm^{-1} and H NMR investigation showed a molecular mass of 7.6 kDa for purified mecasermin. These observations are indicative of proper folding mecasermin with α helical structure and no existence of B sheets in structure of our purified product. Our FTIR results have spectral similarity to the results of wolf and coworkers (12). Through this novel empirical work we obtained the pure mecasermin with higher solubility and refolding yield.

CONCLUSION

Here, we succeeded in developing a novel and efficient method of cell lysis to improve downstream purification steps of mecasermin such as solubilization and refolding. Our findings have also provided valuable data aiming to delineate a simple improved method to attain of high soluble mecasermin with monomeric structure and high purity. Here we opened a new horizon into the effect of cell disruption methods on the further purification process of IB formed mecasermin.

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