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Microwave assisted solubilization of inclusion bodies

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Abstract

Background: Production of recombinant proteins in bacterial hosts often produces insoluble intracellular particles called inclusion bodies. Recovery of active protein from inclusion bodies generally requires their solubilization in chemical denaturants followed by a refolding strategy. The solubilization is carried out with shaking/stirring and takes several hours.

Results: Using inclusion bodies of seven diverse kinds of recombinant proteins [mutants of controller of cell division or death protein B (CcdB), human CD4D12, thioredoxin fusion protein (malETrx), mutants of maltose binding protein (MBP), single chain variable fragment (ScFv) b12 and single chain antigen binding fragment (ScFab) b12 (anti-HIV-1)], it is shown that exposure to microwave irradiation (200 W) for 2 min, solubilized these inclusion bodies completely. This was confirmed by data based upon turbidity measurements at 400 nm and dynamic light scattering studies. These solubilized inclusion bodies could be refolded correctly in all the cases by known methods. The refolding was confirmed by fluorescence emission spectra and biological activity studies.

Conclusion: Solubilization of the inclusion bodies before refolding is a part of protein production processes for several recombinant proteins which are overexpressed in the bacterial host systems. Our results show that microwave assistance can considerably shorten the process time.

Keywords: Solubilization of inclusion bodies, Protein refolding, Microwave assisted reactions, Maltose binding protein, Thioredoxin

Background

Production of recombinant proteins in bacterial hosts often produces insoluble particles called “inclusion bodies” [1-3]. Recovery of active soluble proteins from these inclusion bodies involves two steps. The first step is the solubilization of the inclusion bodies which involves unfolding of the protein molecules by chemical denaturants [4] and the second step is the refolding step. No single refolding method is universally applicable and hence a large number of strategies have been described in the literature for refolding purposes [2,5-8]. The innovations in the first step have been rather limited [9]. Urea and Guanidinium hydrochloride (GuHCl) are the most frequently employed denaturants [10,11]. Some surfactants like cetyltrimethylammonium bromide (CTAB) and sodium dodecyl sulfate (SDS) have been also tried [11-13]. Use of cetyltrimethylammonium chloride (CTAC) has been reported to improve subsequent yields of the refolded proteins [14].

The solubilization step generally requires from 1 to 80 hours [15] and hence constitutes a significant component of process time for production of recombinant proteins [15]. The present work outlines a simple, efficient and fast solubilization procedure. The strategy involves microwave assisted solubilization and takes only 2 minutes.

Microwave assistance is known to facilitate numerous chemical reactions [16], biochemical reactions [17-19], biochemical conjugation [20,21] and protein degradation in proteomics [19,20]. Its application for facilitating rapid solubilization of inclusion bodies does not seem to have been described so far. It is shown that the method works well with inclusion bodies of several diverse kinds of recombinant proteins. It is also shown that the refolded proteins obtained from microwave assisted solubilized inclusion bodies are structurally identical to refolded proteins obtained after conventional and longer solubilization steps. The refolded proteins have been

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characterized by fluorescence emission spectra and biological activity assays.

Results and discussion

The inclusion bodies of MBP224D, MBP264D, CcdB-M97K, malETrx, ScFv b12, ScFab b12, and CD4D12 were used for the development of this method. The properties and refolding of these recombinant proteins by a simple precipitation method have been described recently [7]. These chosen proteins were of diverse nature in terms of size, number of disulfide bridges, proneness to aggregation etc. and hence constitute a good representative selection for testing the generic nature of the microwave assisted solubilization method as well.

Turbidity measurements at 400 nm are generally used for following the extent of solubilization of inclusion bodies [22]. Figure 1 shows the turbidity (as measured at 400 nm) of these various inclusion bodies suspended in three common solubilization reagents (urea, GuHCl, SDS) [10-12] and exposed to microwave irradiation for 2 minutes. For comparison, the turbidity of the inclusion bodies suspended in the aqueous buffer (without any denaturant) is also shown in Figure 1. The drastic reduction in the turbidity after microwave treatment points towards solubilization of the inclusion bodies. Visually, these samples appeared to be clear indicating complete solubilization of the inclusion bodies.

It may be noted that even though the inclusion bodies appeared to have become completely soluble, the turbidity measurements after solubilization (Figure 1) still had some finite values. This result is in agreement with the one reported by Vincentelli [22] with a solubilized protein in 6 M GuHCl.

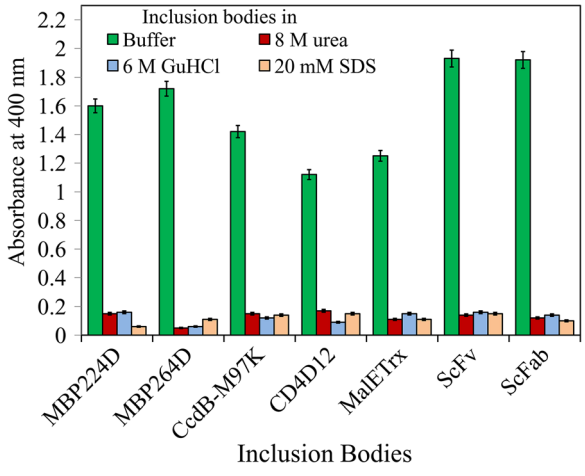

Figure 1 The turbidity measurements at 400 nm of inclusion bodies solubilized by microwave treatment with different denaturants. The inclusion bodies were solubilized by microwave treatment for 2 minutes in 8 M urea, 6 M GuHCl and 20 mM SDS (solutions of all the denaturants were prepared in 50 mM Tris–HCl, pH 7.0) and the absorbance at 400 nm was recorded and compared with the absorbance of inclusion bodies suspended in buffer (50 mM Tris–HCl, pH 7.0) only without any microwave treatment. All the turbidity measurements were carried out at 400 nm on a Beckman Coulter DU730 spectrophotometer.

However, no explanation was offered presumably since the turbidity value was considered insignificant. We decided to investigate this further so as to be sure that the microwave treatment is resulting in complete solubilization of the inclusion bodies.

Table 1 shows the results of the DLS (dynamic light scattering) measurement on the average diameter of the species present in the solutions of 8 M urea, 6 M GuHCl and 20 mM SDS in the aqueous buffers. These values, larger than expected for these denaturant molecules, indicate clustering or aggregation of these molecules in the solution. In fact, this phenomenon is well known and extensive studies of association of these small molecules in aqueous solutions have been carried out [23-26]. It is also interesting to observe that the microwave exposure (identical conditions as used for protein solubilization) disrupted these associations (Table 1). Except in the case of 8 M urea, this microwave treatment caused extensive dissociation of these clusters or aggregates.

Table 2 gives the data on the average diameter obtained before and after solubilization with microwave treatment in the three denaturants for different inclusion bodies. This data is in broad agreement with the turbidity data (Figure 1).

To further ensure that the microwave treatment (for 2 minutes) has resulted in solubilized or unfolded protein molecules which were similar (if not identical) to protein molecules obtained by conventional solubilization (in 24 hours), the average diameter (measured by DLS) of MBP264D after the solubilization procedures were compared (Figure 2). These values obtained with the three denaturants in both the cases are higher than for unfolded proteins or denaturant molecules (Table 1). This is understandable in view of well known interactions between protein molecules and these denaturants [11]. The differences between the average diameters obtained after the two

Table 1 Average sizes of different denaturants

Denaturant	Average diameter (nm) before microwave treatment	Average diameter (nm) after microwave treatment
8 M urea	706	587
6 M GuHCl	110	9
20 mM SDS	422	7

The denaturants 8 M urea, 6 M GuHCl and 20 mM SDS were prepared in 50 mM Tris–HCl, pH 7.0 and their average sizes (nm) were recorded before and after microwave treatment (for 2 minutes), by dynamic light scattering. This was done as a control experiment. The temperature during microwave solubilization was maintained at 26 ± 1°C. The readings were taken in triplicates and the difference among the individual readings was less than 5%.

Table 2 Effect of microwave treatment on the average sizes of different inclusion bodies dissolved in different denaturants

Inclusion Bodies	Average diameter (nm) of inclusion bodies in buffer before microwave treatment	Average diameter (nm) after microwave treatment.		
		8 M urea	6 M GuHCl	20 mM SDS
MBP224D	1493	390	451	130
MBP264D	1504	440	326	130
CcdB-M97K	751	93	438	62
CD4D12	1040	284	338	295
MalETrx	731	120	410	195
ScFv	1444	615	587	560
ScFab	1400	884	811	547

Inclusion bodies of MBP224D, MBP264D, CcdB-M97K, CD4D12, malETrx, ScFv and ScFab, were dissolved in 8 M urea, 6 M GuHCl and 20 mM SDS and subjected to microwave treatment for 2 minutes and the average diameter was recorded. The average diameters of inclusion bodies suspended in 50 mM Tris–HCl, pH 7.0 (without denaturant) and prior to microwave treatment were also recorded for comparison. The average sizes of the dissolved inclusion bodies were measured by dynamic light scattering. In all the measurements the buffer used was 50 mM Tris–HCl, pH 7.0. The temperature during microwave solubilization was maintained at 26 ± 1°C. Protein concentration in all the cases was 2.0 mg.mL^{-1}. The readings were taken in triplicates and the difference among the individual readings was less than 5%.

solubilization procedures are also not entirely unexpected in view of the effect of microwave on dissociation of the denaturant molecules alone (Table 1).

The kinetics of solubilization of inclusion bodies of MBP264D was followed by DLS measurements (Figure 3). The data confirms that minimum of 2 minutes were required at the chosen microwave power to solubilize inclusion bodies. As mentioned before, the inclusion bodies formed clear solutions at this point. The size remained the same even after 2 minutes.

The microwave solubilized malETrx (thioredoxin fusion protein with signal peptide of maltose binding protein) was refolded by affinity precipitation by using Eudragit L-100 as a smart polymer [7]. It was also refolded to a similar structure as the earlier one [7] solubilized by 8 M urea, as shown by the fluorescence emission spectra (Figure 4A) with λ_{max} of 342 nm and insulin aggregation assay (Table 3). CcdB-M97K was also refolded by using Eudragit L-100 [7] and was found to be refolded as shown by the fluorescence emission spectra (Figure 4B). Similarly microwave solubilized inclusion bodies of MBP264D were refolded by affinity precipitation with cationic starch as a smart polymer [7]. The refolded protein was identical to the protein resolubilized without microwave treatment as also described earlier [7], as shown by the fluorescence emission spectra and red shift as well as quenching of the intrinsic fluorescence spectra upon maltose binding (Figure 5) [27].

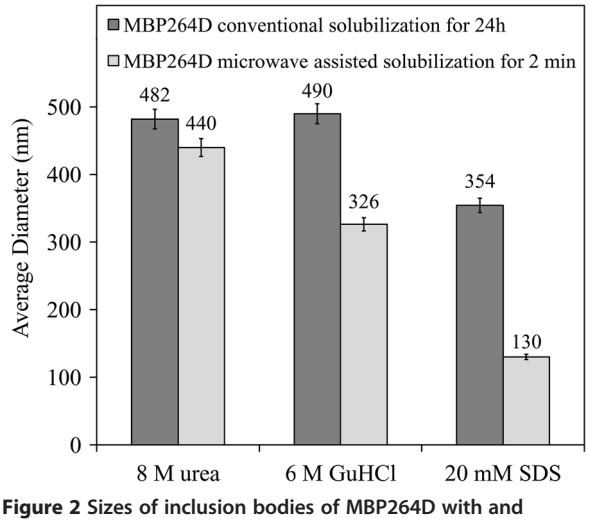

Figure 2 Sizes of inclusion bodies of MBP264D with and without microwave assistance. The inclusion bodies were dissolved in different denaturants in 24 hours at 25°C. The same dissolution was achieved with microwave treatment in 2 minutes. The average sizes of the dissolved inclusion bodies were measured by dynamic light scattering. Solutions of all the denaturants were prepared in 50 mM Tris–HCl, pH 7.0.

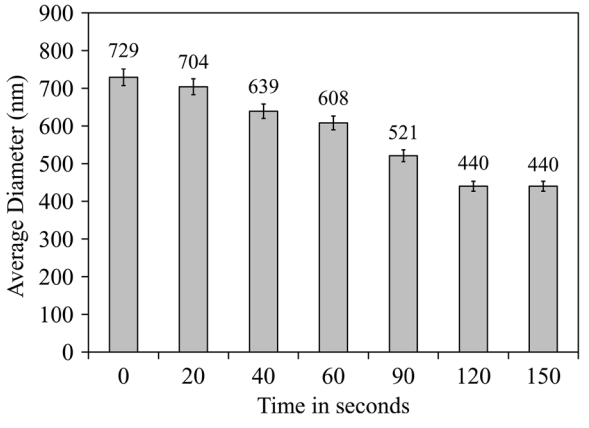

Figure 3 Sizes of inclusion bodies of MBP264D dissolved in 8 M urea with microwave treatment. The inclusion bodies were dissolved in 8 M urea with microwave treatment for varying time intervals. The average sizes of the dissolved inclusion bodies were measured by dynamic light scattering. Solutions were prepared in 50 mM Tris–HCl, pH 7.0.

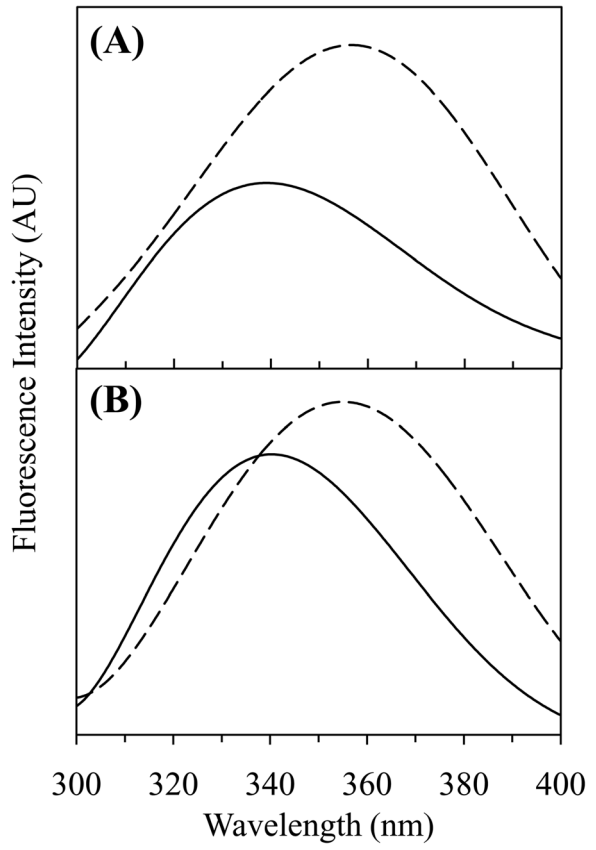

Figure 4 Fluorescence emission spectra of refolded proteins. The inclusion bodies were solubilized by microwave assistance in 2 min with 8 M urea before the refolding process. All the spectra were recorded with 1 μM protein concentration in 10 mM Tris–HCl, pH 7.5, with excitation at 280 using excitation and emission slit widths of 2 nm and 5 nm, respectively: (**A**) Refolded malETrx (thioredoxin) and (**B**) refolded CcdB-M97K. In both the figures, the two curves show: refolded protein (—) and unfolded protein in 8 M urea (– – –).

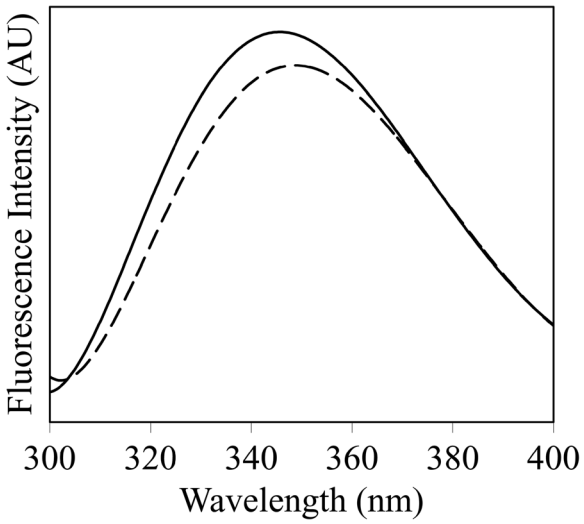

Figure 5 Fluorescence based maltose binding assay for refolded MBP264D. The inclusion bodies were solubilized by microwave assistance in 2 min with 8 M urea before refolding by using cationic starch as a smart polymer. Fluorescence emission spectra (AU, arbitrary units) were acquired in 10 mM Tris–HCl, pH 7.5; using excitation and emission slit widths of 2 nm and 5 nm, respectively. Protein samples at a concentration of 3.5 μg/mL^{-1} were incubated in Tris buffer (—) and in Tris buffer containing 0.1 mM maltose (– – –) 15 min before spectral acquisition.

Figure 6 shows the changes in the inclusion bodies during solubilization as seen by a light microscope. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) techniques could not be used since the denaturants were found to crystallize out during the sample preparation by the standard protocol. Figure 6A shows the inclusion bodies of MBP264D suspended in the aqueous buffer. Figure 6B shows the way the inclusion bodies looked immediately after adding 8 M urea. Figure 6C shows partial solubilization after 30 seconds of microwave treatment. Figure 6D shows that the solubilization was practically complete after 2 minutes. This data further confirms that minimum treatment of 2 minutes by microwave irradiation in a domestic microwave oven led to the complete solubilization of inclusion bodies of several different proteins by the denaturants.

Table 3 Insulin aggregation assay for thioredoxin proteins

	Protein (mg)	Activity (Units)*	Specific-Activity (Units mg^{-1})
Initial protein taken for refolding (solubilized inclusion bodies)	0.50	-	-
Affinity precipitation refolded malETrx	0.32	1.10	3.4
WT-Thioredoxin (Sigma-Aldrich)	-	-	4.0

The experiments were carried out in triplicates and the difference in the individual readings was less than 5%.
*One unit will cause a ΔA650 of 1.0 in 1 min at 25°C.

Experimental

Materials

Ampicillin, isopropyl-β-D-thiogalactopyranoside (IPTG), phenylmethanesulfonylfluoride (PMSF) and guanidinium hydrochloride (GuHCl) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA) and sodium dodecyl sulfate (SDS) were obtained from Merck (Mumbai, India). Urea (PlusOne grade) was a product of GE Healthcare (Uppsala, Sweden). All other reagents used were of analytical grade.

Over-expression of proteins in *E.coli* and isolation of inclusion bodies [7]

E. coli BL21 (DE3) was used for protein expression of malETrx, human CD4D12, mutants of MBP, ScFv b12 and ScFab b12. *E. coli* CSH501 was used for expressing the CcdB mutant. The plasmids used for expression of

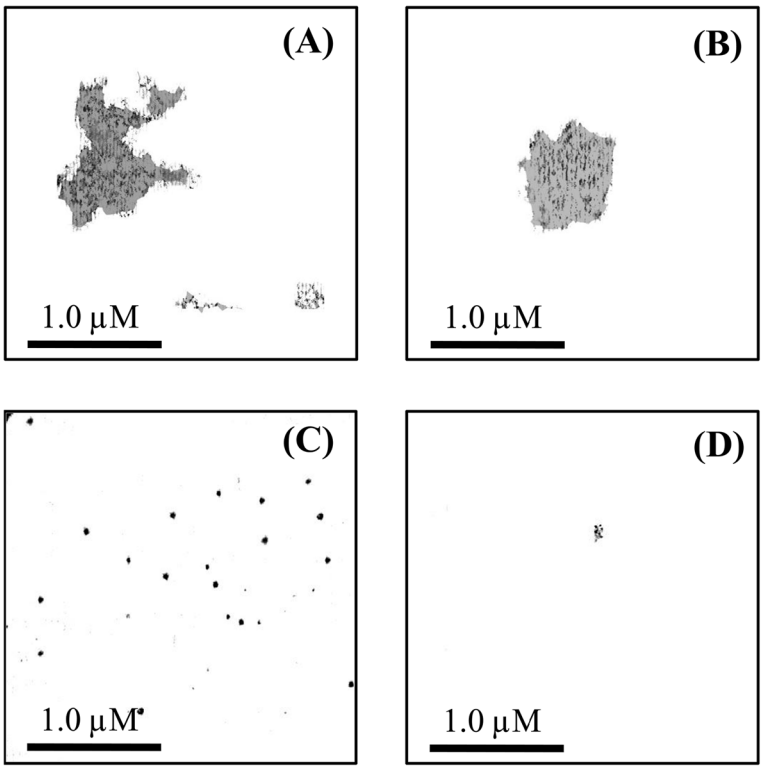

Figure 6 Light microscope images of inclusion bodies of MBP264D. (**A**) inclusion bodies suspended in 50 mM Tris–HCl, pH 7.0, without any treatment, (**B**) immediately after adding 8 M urea, (**C**) subjected to microwave treatment for 30 seconds in 8 M urea and (**D**) microwave treatment for 60 seconds in 8 M urea. All the images are zoomed images taken on a light microscope (Model: CX21i, Olympus) at 1000× magnification with immersion oil.

these proteins were pBAD24 containing CcdB-M97K, MBP224D and MBP264D inserts, pET20b(+) containing (A14E)malETrx insert, pET28a containing human CD4D12 insert, pET22b(+) containing ScFv b12 insert and pComb containing ScFab b12 insert. The plasmid pBAD24 expressing CcdB mutant M97K was transformed into *E. coli* CSH501 [28]. A single colony was picked and inoculated into 5 mL LB medium containing 100 μg mL^{-1} ampicillin. One percent of primary inoculum was transferred into 1 L fresh LB broth (amp+) and grown at 37°C with shaking at 200 rpm until absorbance at 600 nm reached 0.8 and then the induction was carried out. This procedure was repeated for the transformation of the plasmid pET20b(+) containing (A14E) malETrx insert (showing leaky expression), pBAD24 containing MBP224D and 264D inserts, pET22b(+) containing ScFv b12 insert and pComb containing ScFab b12 insert into *E. coli* BL21 (DE3). The plasmid pET28a expressing CD4D12 was transformed into *E. coli* BL21 (DE3) and 50 μg mL^{-1} kanamycin was used as the selection marker. Induction was carried out by adding L-arabinose (0.2%) in case of CcdB-M97K, MBP224D and MBP264D; 0.5 mM IPTG (final concentration) in case of malETrx and CD4D12; and 1 mM IPTG (final concentration) in case of ScFv b12 and ScFab b12, and the culture was further grown under similar conditions for 12 hours at 37°C with shaking at 200 rpm. Cells were harvested, sonicated in resuspension buffer (for CcdB mutant, 50 mM Tris–HCl, pH 8.0/1 mM EDTA/10% glycerol/200 μM PMSF; for malETrx, MBP mutants, ScFv b12 and ScFab b12, 50 mM Tris–HCl, pH 7.0/150 mM NaCl/1 mM EDTA/100 μM PMSF; for CD4D12, 50 mM PBS, pH 7.4/100 μM PMSF) 10 times with 30 seconds pulses on ice, and centrifuged at 9000 × g for 30 minutes at 4°C. The inclusion body pellet was washed (thrice) with washing buffer (50 mM PBS, pH 7.4/0.5% Triton X-100) and centrifuged at 9000 × g for 30 minutes at 4°C.

Inclusion bodies solubilization with microwave

The inclusion bodies of MBP224D, MBP264D CcdB-M97K, CD4D12, malETrx, ScFv and ScFab were suspended in solutions of different denaturants (which included 8 M urea, 6 M GuHCl and 20 mM SDS) in 50 mM Tris–HCl, pH 7.0 in a final volume of 3 mL in an open glass vial and microwave treatment was carried out by placing the reaction mixture inside a domestic microwave oven (Model: NN-K543WF, Panasonic) along with a glass beaker containing 100 ml of water. This was done to

avoid overheating of samples. To maintain the temperature, the samples were exposed for 10 seconds in a microwave and then kept outside for 10 seconds in an ice bath. The reaction mixture was exposed to microwave radiations at low power (200 W) with temperature maintained between 25 and 30°C.

Turbidity measurements

The turbidity at 400 nm was measured by Beckman Coulter DU730 spectrophotometer [22].

Dynamic light scattering (DLS) measurements

Dynamic light scattering measurements were performed at 25°C in laser-spectroscatter 201 by RiNA GmbH (Berlin, Germany). Data analysis was done using PMgr v3.01p17 software supplied with the instrument. The average diameter of the suspension of inclusion bodies was recorded before and after solubilization. The solubilization was carried out both by microwave irradiation for 2 minutes and conventional procedure which consisted of suspending the inclusion bodies in 8 M urea and incubating these at 25°C on a shaker at 200 rpm for 24 hours [15].

Estimation of protein concentration

The protein concentration in all the cases was estimated by the dye binding method using bovine serum albumin as the standard protein [29].

Fluorescence measurements

Fluorescence spectra of the refolded proteins were recorded on a Cary Eclipse, Varian spectrofluorimeter (Mulgrave, Australia) at 25°C using a 1-cm cuvette. Typically, 0.5-1.0 μM protein in 10 mM Tris–HCl, pH 7.5, was used and the fluorescence emission spectra were recorded from 300 nm to 400 nm upon excitation at 280 nm. The excitation and emission slit widths were kept at 2 nm and 5 nm, respectively. All fluorescence spectra were normalized and corrected for buffer contributions.

Assay for thioredoxin

The activity of thioredoxin was assayed by the insulin aggregation assay [30].

Binding assay for MBP

The binding of maltose to MBP was assayed fluorimetrically by observing a red shift and quenching in the intrinsic tryptophan of MBP upon maltose binding [27].

Microscopic examination

Images were taken on a light microscope (Model: CX21i, Olympus) microscope with an Olympus camera attached to the microscope. The samples were viewed under 1000X magnification (100X objective lens and 10X eye piece) which was obtained by using immersion oil.

Refolding of solubilized inclusion bodies

The solubilized inclusion bodies were refolded by using smart polymers as described previously [7]. The essential steps were as follows:

Different aliquots of solubilized inclusion bodies were incubated with 0.2 mL of 2% (w/v) Eudragit L-100 (final concentration, 0.2%, w/v) for CcdB-M97K and malETrx and 0.3 mL of 2% (w/v) cationic starch (final concentration, 0.3%, w/v) for MBP264D, and the final volume was made up to 2 mL with 50 mM Tris–HCl, pH 7.5. The final protein concentration was 0.2-2.5 $mg.mL^{-1}$. After incubation at 25°C for 1 hour with shaking at 200 rpm, the polymer-protein complex was precipitated by lowering the pH to 4.0 with 2 M acetic acid in case of Eudragit L-100 and by the addition of 10% (w/v) PEG and 50 mM $CaCl_2$ [stock solutions of PEG (40%, w/v) and $CaCl_2$ (1 M) were made in distilled water] for cationic starch. The precipitate was separated from the unbound protein in the supernatant by centrifugation (10000× *g*, 10 minutes) at room temperature. The precipitate was then washed twice with 0.01 M acetate buffer, pH 4.0 for Eudragit L-100 and 50 mM Tris–HCl, pH 7.5 for cationic starch. The bound protein was dissociated from the polymer by suspending the polymer-protein complex in 70% (v/v) ethylene glycol solution made in 50 mM Tris–HCl, pH 7.5 for Eudragit L-100 and chilled 1 M NaCl (in 50 mM Tris–HCl, pH 7.5) for cationic starch and incubating at 4°C for 1 hour with shaking at 150 rpm. The supernatant collected after centrifugation at 10000× *g* for 10 minutes at 4°C, was used for spectroscopic measurements and activity assays. All measurements were carried out after removal of the dissociating agent by membrane filtration (Amicon Ultra-15 3K, Millipore).

Conclusions

Microwave irradiations cause continuous realignment of the polar molecules with the changing field. This causes solutions containing polar molecules (such as water) to get heated. However, as opposed to simple heating, the energy input is more efficient and happens over a shorter period of time. Hence, many workers describe such effects as “non-thermal effects” of the microwave irradiations. Hence, exposure to microwave irradiations is known to accelerate many processes [16,17].

The results obtained with seven inclusion bodies of diverse kinds of proteins show that a minimum exposure to microwave radiation for just 2 minutes is enough to solubilize inclusion bodies irrespective of which of the commonly used denaturants is used. These results have been obtained with a commonly available ordinary domestic

microwave oven. Such microwave ovens are available in all biochemistry laboratories. Wherever available, a microwave with a temperature control can also be utilized. The results further indicate the method described here did not merely solubilize the inclusion bodies but led to unfolded protein molecules which could be refolded just as well as those obtained by existing procedures which require longer duration of time. Production of many industrially important proteins (including pharmaceutical proteins) requires solubilization of inclusion bodies followed by a refolding step. The method outlined here would make the whole process shorter and possibly more economical. As much as the use of microwave in case of conventional methods is considered a strong component of green chemistry, the method described here can be considered as a part of initiative to develop greener production processes [31].

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
ID and SG carried out the experimental work. MNG designed the study and was involved in all discussions, interpretation of data and writing the manuscript. All authors read and approved the final manuscript.

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