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Smart polymer mediated purification and recovery of active proteins from inclusion bodies

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ABSTRACT

Obtaining correctly folded proteins from inclusion bodies of recombinant proteins expressed in bacterial hosts requires solubilization with denaturants and a refolding step. Aggregation competes with the second step. Refolding of eight different proteins was carried out by precipitation with smart polymers. These proteins have different molecular weights, different number of disulfide bridges and some of these are known to be highly prone to aggregation. A high throughput refolding screen based upon fluorescence emission maximum around 340 nm (for correctly folded proteins) was developed to identify the suitable smart polymer. The proteins could be dissociated and recovered after the refolding step. The refolding could be scaled up and high refolding yields in the range of 8 mg L^{-1} (for CD4D12, the first two domains of human CD4) to 58 mg L^{-1} (for malETrx, thioredoxin fused with signal peptide of maltose binding protein) were obtained. Dynamic light scattering (DLS) showed that polymer if chosen correctly acted as a pseudochaperonin and bound to the proteins. It also showed that the time for maximum binding was about 50 min which coincided with the time required for incubation (with the polymer) before precipitation for maximum recovery of folded proteins. The refolded proteins were characterized by fluorescence emission spectra, circular dichroism (CD) spectroscopy, melting temperature (T_m), and surface hydrophobicity measurement by ANS (8-anilino1-naphthalene sulfonic acid) fluorescence. Biological activity assay for thioredoxin and fluorescence based assay in case of maltose binding protein (MBP) were also carried out to confirm correct refolding.

1. Introduction

While it is well established that a correctly folded conformation of a protein – called the native structure – is responsible for its biological activity [1], the exact mechanisms are still less than completely understood. The well known work by Anfinsen showed that the information for folding resides in the primary sequence of the protein [2]. As Hartl et al. [3] recently observed "Although small proteins may fold at very fast speeds (within microseconds), in dilute buffer solutions, larger multidomain proteins may take minutes to hours to fold, and often even fail to reach their native states in vitro". In vivo, protein crowding [4] contributes to aggregation of non-native structures. This is prevented by molecular chaperones or chaperonins in a cell. Their role is not always limited to prevention of aggregation, but may extend to acceleration of folding and reversal of misfolding events [3]. Many excellent reviews are available on protein folding [5,6]. While Sinha and Udgaonkar [5] have provided a rigorous treatment of early events in protein folding, Nickson and Clarke [6] have reviewed both theoretical and experimental methods (and their results) used to study protein folding. There is enough evidence that protein folding involves existence of one or more partially folded structures. In many cases, it is possible to isolate 'molten globules' which occur on the folding pathway. The 'oil drop' model of protein structure envisages that there is a hydrophobic core with polar amino acids on the surface H-bonded with water. Hydrophobic clusters do occur on the protein surface and are quite often part of a specific binding site for ligands/substrates. Apart from the above 'nucleation model', 'energy landscape model' has also been proposed more recently, where folding intermediates are viewed as 'kinetic traps' on the folding pathway. The greater understanding of protein folding is also of practical utility in the context of protein refolding. The overexpression of recombinant proteins in bacterial hosts often leads to the formation of inactive and insoluble aggregates called inclusion bodies. In some cases, proteins in these inclusion bodies may not be completely inactive [7]. Protein aggregation as such has also attracted attention as the cause behind several neurodegenerative diseases and cataract formation

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[8]. From the biotechnological perspective, the recovery of soluble active proteins from inclusion bodies generally involves: (1) solubilization of the inclusion bodies by denaturants to obtain the protein in an unfolded form, (2) a second step of refolding [9–11]. While a large number of strategies have been described in the literature for the refolding step [9,11–14], it is a "no single shoe fits all" situation.

Tsumoto et al. [12] have provided an excellent overview of different classes of additives which have been used during protein refolding. These can be classified as folding enhancers (e.g. sucrose, ammonium sulfate) or aggregation suppressors (e.g. mild denaturants or low concentration of denaturants like urea or guanidine hydrochloride). The use of PEG in assisting protein refolding is a pioneering study in the area. While its detailed role on the basis of thermodynamics has been discussed [15], it essentially binds to hydrophobic regions of the folding intermediate(s), prevents protein–protein interaction and hence essentially works as an aggregation suppressor. Use of smart polymers as "pseudochaperonins" for refolding has been described by various workers as one such approach [16–19]. Smart polymers are reversibly soluble–insoluble materials which respond to various stimuli such as pH changes, temperature changes and presence of different chemical species [20–25]. Use of smart polymers (as compared to other water soluble polymers like PEG) offers the added advantage that one can easily isolate the folded protein–polymer complex as a precipitate by applying a suitable stimulus. This precipitation often will also result in simultaneous purification of the desired protein [21,26,27]. In the past, refolding by smart polymers has been generally limited to working with chemically or thermally denatured proteins [16,28,29]. A few years back, we reported use of a pH-responsive methyl methacrylate polymer for obtaining active recombinant controller of cell division or death B (CcdB) protein from its inclusion bodies [19]. Unfortunately, refolded CcdB could not be dissociated from that polymer Eudragit S-100.

In this work, we have attempted to develop the use of smart polymers as a general approach for recovery of active proteins from their inclusion bodies. Obviously, a single smart polymer would not work with different proteins. So, we decided to develop a screen for the searching of a suitable smart polymer in a 96-well plate format. Our results show that, at least with a variety of proteins investigated by us, it was possible to identify a suitable smart polymer for obtaining a reasonable recovery of the active protein by refolding. The refolding was confirmed by biological activity (wherever possible), fluorescence, and circular dichroism (CD) spectroscopy. The proteins used for refolding from inclusion bodies in the present study are: five aggregation prone mutants of the *E. coli* proteins-controller of cell division or death B (CcdB) [30], maltose binding protein (MBP) [31], and thioredoxin fused with signal peptide of MBP (malETrx) [13]; the first two domains of human CD4 (CD4D12) [32]; single chain variable fragment (ScFv) b12 and single chain antigen binding fragment (ScFab) b12, both derived from the anti-human immunodeficiency virus (HIV)-1 antibody b12, which binds to the CD4 binding site on gp120 of HIV-1 [33].

2. Materials and methods

2.1. Materials

Eudragit L-100 and S-100 were products of Rohm Pharma GmbH (Weiterstadt, Germany). This is a copolymer of methacrylic acid and methyl methacrylate (in a molar ratio of 1:1) with average molecular weight of 1,35,000 g/mol (Product sheet, Rohm Pharma). Cationic starch (Catamyl-VS; 99.6% purity), starch derivatized with a quaternary ammonium compound, was a kind gift from Chemtech Marketing (Delhi, India). Protanal LF 10/60 (free alginate from brown seaweed) having a high content of guluronic acid (65–75%) was a product of Protan A/S (Drammen, Norway). The average molecular weight of Protanal LF 10/60 is 3,20,000 g/mol [34]. Alginic acid, composed predominantly of mannuronic acid residues (catalog no. A-2158), 8-anilino1-naphthalene sulfonic acid (ANS), phenylmethanesulfonylfluoride (PMSF), isopropyl β-D-thiogalactopyranoside (IPTG) and ampicillin were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade.

2.2. Strains and expression plasmids

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 wild type (WT) CcdB and its mutants. The plasmids used for expression of these proteins were pBAD24 containing CcdB-F17P, CcdB-M97K, MBP224D and MBP264D inserts, pET20b(+) containing (A14E)malETrx insert, pET28a containing human CD4D12 insert, pET22(+) containing ScFv b12 insert and pComb containing ScFab b12 insert.

2.3. Overexpression in E. coli

The plasmid pBAD24 expressing CcdB mutants F17P or M97K, was transformed into *E. coli* CSH501 [30]. 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 OD_{600} reached 0.8. Induction was carried out by adding L-arabinose (0.2%) and the culture was further grown under similar conditions for 12 h at 37 °C at 200 rpm. This procedure was repeated for the transformation of the plasmid pET20b(+) containing (A14E) malETrx insert (showing leaky expression), pBAD24 containing MBP 224D and 264D inserts, pET22(+) 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 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 h.

2.4. Isolation and solubilization of inclusion bodies

Cells were harvested, sonicated in resuspension buffer (For CcdB mutants, 50 mM Tris/pH 8.0/1 mM EDTA/10% glycerol/200 mM PMSF; for malETrx, MBP mutants, ScFv b12 and ScFab b12, 50 mM Tris/pH 7.0/150 mM NaCl/1 mM EDTA/100 mM PMSF; for CD4D12, PBS/pH 7.4/100 mM PMSF) 10 times with 30 s pulses on ice, and centrifuged at 9000 × *g* for 30 min 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 min. Isolated inclusion bodies were solubilized with 8 M urea in 50 mM Tris–HCl buffer (pH 7.5 for CcdB mutants, malETrx, CD4D12 and MBP mutants and pH 7.0 for ScFv b12 and ScFab b12) containing 100 mM DTT and incubated with stirring for 5 h at room temperature.

2.5. Preparation of Eudragit solution

Eudragit solutions (2%, w/v) for both kinds of Eudragit (L-100 and S-100) were prepared by suspending the polymer powder (2 g) in 50 mL of 50 mM Tris–HCl buffer, pH 7.5. The pH of the solution was raised to 11.0 with 3 M NaOH and stirred until the polymer dissolved. The pH was then readjusted to 7.5 with 3 M HCl, and the volume of the solution was increased to 100 mL with buffer.

Eudragit solutions thus prepared were stored at 4 °C. All the experiments were done with these stored polymer solutions.

2.6. Preparation of cationic starch solution

Cationic starch solution (2%, w/v) was prepared by adding 0.2 g of starch in 10 mL of distilled water and heating the suspension at 70 °C for about 2–3 min till a clear solution was obtained. The solution was cooled to room temperature and diluted appropriately with 50 mM Tris–HCl buffer, pH 7.5, to get the desired concentration wherever required.

2.7. Preparation of alginate solution

Alginate solutions (2%, w/v) for both kinds of alginate (Protanal LF and Alginic acid) were prepared by dissolving 0.4 g of dry alginate in 20 mL of water and the pH was adjusted to 7.0. The solutions were stored at 4 °C and diluted with appropriate buffer for further use.

2.8. High-throughput screening of the affinity ligand (smart polymer) in 96-well plate format

An aliquot of solubilized inclusion bodies containing 0.1 mg protein (final concentration 0.5 mg mL^{-1}) was taken in the 96-well plate along with the polymer (final concentration of the polymer was 0.2%, w/v, for Eudragit L-100 and S-100, and 0.5%, w/v, for alginate and other polymers) and the volume was made up to 0.2 mL with 50 mM Tris–HCl buffer pH 7.5 in the 96-well plate. Six different polymers were examined for refolding of different inclusion body proteins. In the dilution control, appropriate buffer was added instead of the smart polymer. The 96-well plate was incubated at 25 °C for 1 h at 150 rpm. The appropriate polymer was identified by measuring the fluorescence emission maximum (λ_{max}) with excitation at 280 nm, of the desired wells in 96-well plate placed in a microtitre plate reader accessory of the Cary Eclipse spectrofluorimeter (Varian Inc., Victoria, Australia) at 25 °C.

2.9. Refolding of two mutants of CcdB (F17P and M97K), malETrx and CD4D12 from inclusion bodies with Eudragit L-100

Different aliquots of solubilized inclusion bodies were incubated with 0.2 mL of 2% (w/v) Eudragit L-100 (final concentration of Eudragit was 0.2%, w/v), and the final volume was made up to 2 mL with 50 mM Tris–HCl buffer, pH 7.5, containing 0.1 M KCl and 1 mM EDTA. The final protein concentration was 0.2–2.5 mg mL^{-1}. After incubation at 25 °C for 1 h, the polymer–protein complex was precipitated by lowering the pH to 4.0 with 2 M acetic acid [35]. The precipitate was separated from the unbound protein in the supernatant by centrifugation (10,000 × *g*, 10 min) at room temperature. The precipitate was then washed twice with 0.01 M acetate buffer, pH 4.0. The polymer–protein complex was incubated with different concentrations of dissociating agents (NaCl and ethylene glycol) in 50 mM Tris–HCl buffer, pH 7.5, for 1 h at 4 °C with shaking at 150 rpm. The supernatant collected after centrifugation at 10,000 × *g* for 10 min at 4 °C, was used for spectroscopic measurements and activity assay. All measurements were carried out after removal of the dissociating agent by membrane filtration (Amicon Ultra-15 3K, Millipore).

2.10. Refolding of two mutants of MBP (224D and 264D) from inclusion bodies with cationic starch

Cationic starch is known to bind soluble MBP and MBP fusion proteins [36], therefore cationic starch was used as a pseudochaperonin for the refolding of MBP mutants from their inclusion bodies. Different aliquots of the solubilized inclusion bodies of MBP mutants (224D and 264D) were incubated with 0.3 mL of 2% (w/v) cationic starch (final concentration, 0.3%, w/v) and final volume was made up to 2 mL with 50 mM Tris–HCl buffer, pH 7.5. The final protein concentration was 0.2–2.5 mg mL^{-1}. After incubation at 25 °C, 150 rpm for 1 h, the polymer–protein complex was precipitated 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. Aliquots of the solutions were taken to precipitate the polymer–protein complex] [36]. After 10 min, the precipitate was separated from the supernatant by centrifugation (10,000 × *g*, 10 min at 4 °C). The precipitate was then washed twice with 2 mL of 50 mM Tris–HCl buffer, pH 7.5, at 4 °C. The bound protein was dissociated from the polymer by suspending the polymer–protein complex in 1 mL of chilled 1 M NaCl (in 50 mM Tris–HCl buffer, pH 7.5) and incubating at 150 rpm at 4 °C for 1 h [36]. The polymer was separated from the dissociated protein by centrifugation (10,000 × *g*, 10 min) at 4 °C. Further characterization and activity assays were determined in the supernatant after extensive dialysis against the 50 mM Tris–HCl buffer, pH 7.5 to remove NaCl.

2.11. Refolding of two antibody fragments (ScFv b12 and ScFab b12) from inclusion bodies with alginate

Different aliquots of solubilized inclusion bodies of ScFv b12 and ScFab b12 were incubated with 0.5 mL of 2% (w/v) Protanal LF (final concentration, 0.5%, w/v), and the final volume was made up to 2 mL with 50 mM Tris–HCl buffer, pH 7.0. The final protein concentration was 0.2–2.5 mg mL^{-1}. After incubation at 25 °C for 1 h, the polymer–protein complex was precipitated by the addition of 1 M $CaCl_2$ (final concentration of $CaCl_2$ in solution was 60 mM) [29]. The precipitate was separated from the supernatant by centrifugation (10,000 × *g*, 10 min). The precipitate was then washed twice with 2 mL of 50 mM Tris–HCl buffer, pH 7.0, containing 0.06 M $CaCl_2$. The bound protein was dissociated off of the polymer by suspending the polymer–protein complex in 2 mL of chilled 1 M NaCl (prepared in 50 mM Tris–HCl buffer, pH 7.0) and incubating this suspension at 4 °C for 18 h with shaking at 150 rpm. Further characterization and activity assays were determined in the supernatant after extensive dialysis against the 50 mM Tris–HCl buffer, pH 7.0 to remove NaCl.

2.12. Polyacrylamide gel electrophoresis

SDS-PAGE of the protein samples using 15% gel was performed according to Hames [37], using a Genei gel electrophoresis unit (Bangalore Genei, Bangalore, India). The protein bands on the gel were visualized using Coomassie Blue stain.

2.13. Estimation of protein concentration

The protein concentrations were estimated using extinction coefficients (at 280 nm) of 1.4 mL mg^{-1} cm^{-1} in the case of CcdB [38], 18,450 M^{-1} cm^{-1} for CD4D12 as calculated from the amino acid sequence [39] and 1.46 mL mg^{-1} cm^{-1} for pure MBP [40]. The protein concentration in other cases was estimated by the dye binding method using bovine serum albumin as the standard protein [41].

2.14. Spectroscopic measurements

2.14.1. Fluorescence measurements

All fluorescence spectra were recorded at 25 °C on a Cary Eclipse, Varian spectrofluorimeter. Typically, 1.0–2.0 μM protein in 10 mM Tris–HCl, pH 7.5, was used and the fluorescence emission spectra were recorded from 300 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.

2.14.2. ANS binding measurements

All ANS binding fluorescence measurements were carried out on a Cary Eclipse spectrofluorimeter at 25 °C. Samples were excited at 390 nm, and emission spectra were collected over the wavelength range of 420–600 nm. The excitation and emission slit widths were kept at 2 nm and 5 nm, respectively. The protein and ANS concentrations used were 1 and 100 μM, respectively. All fluorescence spectra were corrected for buffer contributions.

2.14.3. Circular dichroism (CD) measurements

Far-UV CD spectra were recorded on a JASCO J-815 spectropolarimeter (Jasco Corporation, Tokyo, Japan) equipped with a Peltier-type temperature controller and a thermostated cell holder, interfaced with a thermostatic bath at 25 °C using a cell with a path length of 0.1 cm. Typical spectral accumulation parameters were a scanning rate of 50 nm/min with a 2 nm bandwidth over the wavelength range 200–250 nm with six scans averaged for each far-UV spectrum using a protein concentration of 10–15 μM in 10 mM Tris–HCl, pH 7.5. The CD data are presented in terms of mean residue ellipticity (MRE, expressed as deg cm^2 dmol^{-1}) as a function of wavelength, calculated as given below according to the procedure described earlier [42]:

$$[\theta]_{\mathrm{MRE}} = \frac{\mathrm{MRW} \times \theta_{obs}}{10 \times d \times c}$$

where $[\theta]_{\mathrm{MRE}}$ is the calculated mean residue ellipticity (deg cm^2 dmol^{-1}), MRW is the mean residue weight for the peptide bond [MRW is calculated as, MRW = M/N-1, where M is the molecular mass of the polypeptide chain (Da), and N is the number of amino acids in the chain], θ_{obs} is the observed ellipticity (expressed in degrees), d is the pathlength (cm), and c is the protein concentration (g mL^{-1}). All CD spectra were corrected for buffer contributions and secondary structures were calculated by using web based K2d neural network software package (http://www.embl.de/~andrade/k2d.html) [43].

Thermal denaturation curves were determined directly by monitoring the ellipticity changes at 222 nm. Samples with a concentration of 0.2 mg mL^{-1} were used. The temperature of sample solution was raised linearly by 1 °C min^{-1}. The heating curves were corrected for an instrumental baseline obtained by heating the buffer (10 mM Tris–HCl, pH 7.5) alone. The melting temperature (T_{m}) was calculated from the first-order derivatives of the ellipticity-temperature plot.

2.14.4. Dynamic light scattering (DLS) measurements

Dynamic light scattering measurements were performed at 25 °C using the instrument laser-spectroscatter 201 by RiNA GmbH (Berlin, Germany). Data analysis was done using PMgr v3.01p17 software supplied with the instrument. Solubilized inclusion bodies (with a protein concentration of 0.5 mg mL^{-1}) in 10 mM Tris–HCl buffer, pH 7.5, were incubated with smart polymer for different time intervals at 25 °C. The sample (50 μL) was manually injected into a flow cell (1.5 mm path length) and illuminated by a 100 mW, 660 nm laser diode. Prior to measurements, the buffer solutions were filtered through a 0.2 μm filter, while the smart polymer solution was centrifuged at 4000 × g for 15 min and the solubilized inclusion bodies centrifuged at 10,000 × g for 15 min before use.

2.15. Activity assays

2.15.1. Assay for thioredoxin

The activity of thioredoxin was assayed by the insulin aggregation assay [44].

2.15.2. 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 [45].

3. Results

3.1. High-throughput screening of the affinity ligand (smart polymer) in 96-well plate format for appropriate refolding of the proteins

A protein usually binds to a polymer which has the affinity for that particular protein, therefore identifying the appropriate smart polymer becomes a critical step for refolding the protein by the action of these smart polymers (pseudochaperonins). We have developed a high-throughput method for identifying the appropriate smart polymer in 96-well plate format (Table 1). The suitable smart polymer is chosen for appropriate refolding by looking at the fluorescence emission maximum, as given in Table 1.

The various polymers chosen to develop this screen have been described earlier as smart polymers and used for purification of proteins by affinity precipitation [21,27,46]. Eudragit S-100 and L-100 are commercially available synthetic polymers. Both are methyl methacrylate polymers differing in extent of esterification of the carboxyl groups in the polymer chains. Both are pH responsive polymers [19–21]. Protanal LF and Alginic acid are both alginates, again commercially available from different vendors. Alginates are polysaccharides and block copolymers of mannuronic acid and guluronic acid blocks. The two alginates chosen are representative of a wide variety of alginates which occur in nature and differ in their mannuronic acid and guluronic acid content [29,47]. Alginate forms insoluble calcium-alginate in the presence of Ca^{2+}. Cationic starch has been used earlier for purification of MBP and MBP fusion proteins [36]. κ-Carrageenan is again a naturally occurring polysaccharide, which becomes insoluble in the presence of K^+ [48]. Chitosan is a partially deacetylated form of chitin, a naturally occurring polymer of N-acetylglucosamine [23]. Chitosan is a pH-responsive polymer. The choice of these polymers (apart from their ready availability from commercial sources) is based upon two considerations: (1) availability of the earlier experience with their use in protein purification, (2) diverse chemical structure. The chemical structures and some relevant properties of the polymers used are given in Table 2. Affinity precipitation essentially consists of triggering precipitation of polymer–protein conjugate in response to the appropriate stimulus. Here, with no known existence of any affinity between polymer and target protein, it would be more appropriate to call this simply precipitation (however see later discussion on this issue). The design of the screen was to incubate solubilized inclusion bodies of chosen proteins with solutions of all the polymers in different wells (Table 1). After 1 h, fluorescence emission was recorded for each sample. Folded proteins are characterized by the blue shift of the fluorescence emission maximum. Generally, it is around 340 nm for most (tryptophan containing) proteins [49]. These measurements during the screening were made while proteins were still bound to the polymers. Earlier work showed that refolded proteins have the same (or very close) value of $\lambda_{\max}$ (emission) while bound to the smart polymers [19].

The results (Table 1) indicated that:

(a) CcdB-F17P was refolded by both Eudragit L-100 and Eudragit S-100 and by no other polymer.
(b) malETrx and CD4D12 were also refolded by these two polymers.
(c) For ScFv b12 and ScFab b12, Protanal LF was successful in refolding. It is interesting that the analogous polymer Alginic acid was

Table 1
High-throughput screening of the affinity ligand (smart polymer) in 96-well plate for appropriate refolding of the proteins. The promising conditions are shown in bold.

Proteins[a]	1 Inclusion bodies in 8 M urea	2 Dilution control	3 Eudragit L-100	4 Eudragit S-100	5 Protanal LF	6 Alginic Acid	7 Cationic Starch	8 κ-Carrageenan	9 Chitosan
Fluorescence emission maxima (λ_{max})									
A	358 nm	355 nm	**340 nm**	**340 nm**	351 nm	352 nm	354 nm	355 nm	352 nm
CcdB-F17P		(1:0.93)	**(1:0.81)**	**(1:0.82)**	(1:0.91)	(1:0.91)	(1:0.94)	(1:0.94)	(1:0.93)
B	356 nm	352 nm	**342 nm**	**342 nm**	350 nm	352 nm	352 nm	354 nm	351 nm
malETrx		(1:0.80)	**(1:0.50)**	**(1:0.50)**	(1:0.78)	(1:0.80)	(1:0.80)	(1:0.82)	(1:0.77)
C	357 nm	354 nm	**340 nm**	**342 nm**	352 nm	353 nm	355 nm	353 nm	355 nm
CD4D12		(1:0.84)	**(1:0.62)**	**(1:0.63)**	(1:0.82)	(1:0.82)	(1:0.85)	(1:0.82)	(1:0.85)
D	360 nm	353 nm	354 nm	355 nm	**340 nm**	346 nm	352 nm	354 nm	352 nm
ScFv b12		(1:0.90)	(1:0.92)	(1:0.92)	**(1:0.78)**	(1:0.84)	(1:0.90)	(1:0.92)	(1:0.90)
E	360 nm	352 nm	354 nm	355 nm	**340 nm**	345 nm	352 nm	355 nm	355 nm
ScFab b12		(1:0.86)	(1:0.84)	(1:0.86)	**(1:0.72)**	(1:0.78)	(1:0.84)	(1:0.86)	(1:0.84)

[a] Rows 1–9 indicate inclusion bodies in 8 M urea or dilution control of solubilized inclusion bodies with appropriate buffer instead of the smart polymer or solubilized inclusion bodies with different smart polymers and columns A–E indicate different inclusion bodies of different proteins. The numbers in the parentheses below the fluorescence emission maxima (λ_{max}) values indicate the ratio of the fluorescence intensity at that λ_{max} (emission) to the fluorescence intensity of the protein solution in 8 M urea at λ_{max} (emission). The experiments were carried out as described in Section 2.8.

not as good for this purpose. Again, other polymers did not cause refolding.

As dilution can also cause refolding, at least partly in some cases [9,12], a control with the simple addition of buffer without any polymer was also run to assess the contribution of the polymers in refolding (fluorescence spectra of the individual wells not shown).

3.2. Refolding of two mutants of CcdB (CcdB-F17P and M97K) from inclusion bodies

CcdB is a homodimeric protein of 101 amino acids and is encoded by F plasmid [50,51]. CcdB has been of great interest for researchers due to its cytotoxic activity in *E. coli* by the inhibition of DNA gyrase. The mutants of CcdB taken in the present study are highly aggregation prone and form inclusion bodies upon expression, and cannot be refolded by conventional refolding techniques such as dilution or dialysis [13]. Therefore, the refolding of CcdB mutants by Eudragit was investigated further. As earlier work had shown that with Eudragit S-100, it is not possible to dissociate the refolded CcdB from the polymer [19]; Eudragit L-100 was chosen in the present work. Table 3 gives the results on attempts to dissociate the CcdB-F17P protein from the polymer and recover it. Essentially, like in any affinity precipitation work [19,23,28,29], after incubating the polymer–protein complex with the dissociating agent (various concentrations of each were tried), the polymer was precipitated by suitable stimuli and up to 93% protein could be recovered. Experiments showed that CcdB and its mutants can be dissociated from Eudragit L-100. The fluorescence emission spectra of CcdB-F17P along with the polymer (Fig. 1) (at different time intervals during refolding) show: (a) the refolding of all the protein molecules takes place only after the binding event is completed at around 50 min, (b) it is the binding step which is responsible for the refolding and subsequent precipitation does not play any role in refolding and only facilitates recovery of the folded protein in the purified form, (c) the λ_{max} (emission) of 340 nm for the refolded protein at around 50 min indicates that the refolded protein is devoid of any significant aggregates as protein aggregation is known to cause blue shift of the λ_{max} (emission) beyond 340 [19,52]. Dynamic light scattering (DLS) was used to follow the time course of binding of the polymer with the CcdB mutant (Fig. 2A). It is interesting to note that again a minimum of 50 min was necessary for polymer–protein association to reach the maximum size. This also confirmed that the refolding is mediated by actual binding between the protein and polymer. Similarly, solubilized inclusion bodies of ScFv b12 with the appropriate smart polymer Protanal LF, showed a significant increase in size with time, again reaching a maximum size at around 50 min as shown in Fig. 2A.

The conclusion was further confirmed by monitoring interaction of proteins with polymers which were not conducive to refolding based upon the screen (Table 1). It was seen that incubation of proteins CcdB-F17P and ScFv b12 with inappropriate polymers (Protanal LF and Eudragit L-100, respectively), did not lead to any significant interaction as tracked by light scattering (Fig. 2A) i.e. no significant size increase (due to interaction) with time could be seen.

Over the years, the meaning of 'affinity' has undergone drastic changes [53,54]. Any adequate level of interaction in a selective fashion is viewed as an affinity interaction. In that sense, the strategy used here could be, (broadly speaking) called affinity precipitation. The nature of the polymer (Table 2) found suitable for refolding and dissociation of the refolded protein by 70% ethylene glycol (Table 3) indicated that the interaction between the polymer and protein is largely hydrophobic in nature. With isoelectric points

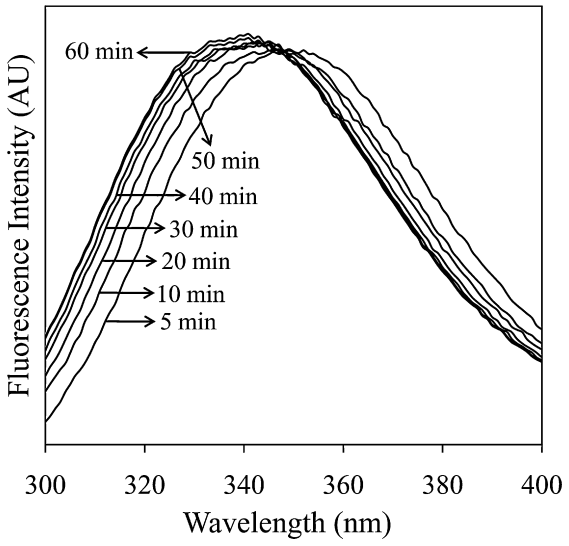
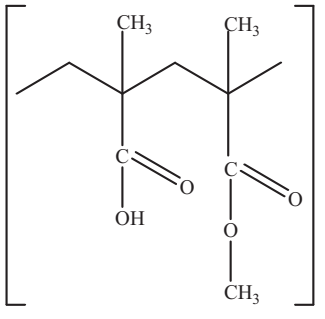
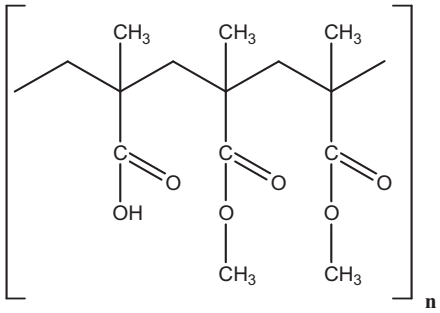
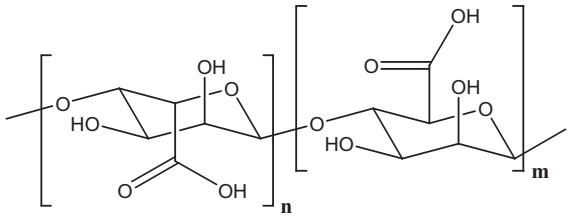
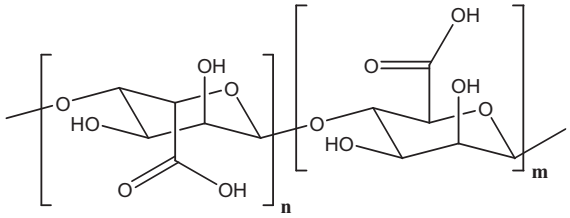
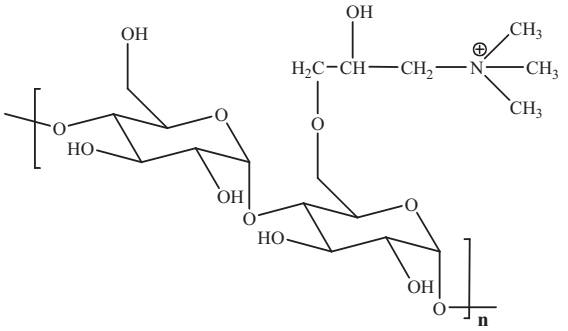
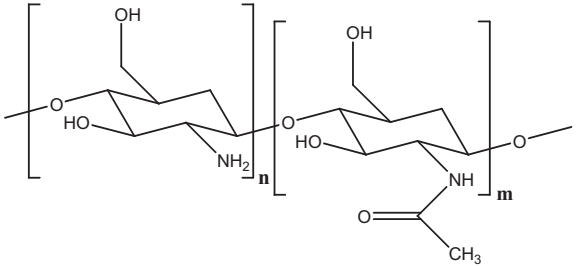
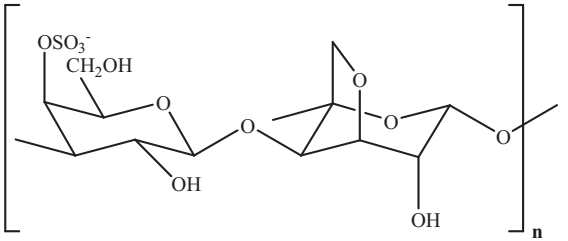


Fig. 1. Fluorescence emission spectra (Arbitrary units, AU) of solubilized inclusion bodies of CcdB-F17P with Eudragit L-100. The Eudragit L-100 concentration was 0.2% (w/v) and the protein concentration was 0.5 mg mL^{-1} in a total volume of 0.2 mL. All the spectra were taken at different time intervals and corrected for buffer contributions in 10 mM Tris–HCl, pH 7.5, using excitation and emission slit widths of 2 nm and 5 nm, respectively.

Table 2
Properties of the different smart polymers used.

S. no.	Smart polymer	Average molecular weight (Da)	Composition	Ionic state at neutral pH	Chemical structure	References
1.	Eudragit L-100	135,000	Copolymer of methacrylic acid and methyl methacrylate (1:1)	Anionic		[Product specification Rohm Pharma][b]
2.	Eudragit S-100	135,000	Copolymer of methacrylic acid and methyl methacrylate (1:2)	Anionic		[Product specification Rohm Pharma][b]
3.	Protanal LF	3,20,000	Copolymer of guluronic acid and mannuronic acid, composed primarily of guluronic acid residues	Anionic		[34]
4.	Alginic Acid	1,00,000	Copolymer of guluronic acid and mannuronic acid, composed primarily of mannuronic acid residues	Anionic		[60]

5.	Cationic Starch	–	Polymer of glucose, derivatized with a quaternary ammonium compound	Cationic		[36]
6.	Chitosan	4,00,000	A linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (80%) and N-acetyl-D-glucosamine (20%)[a]	Cationic		[Product specification Fluka, Sigma–Aldrich (Cat. no. 22742)]
7.	κ-Carrageenan	2,50,000–3,50,000	Polymer composed of 1,3α-1,4β-galactans having one sulfate every disaccharide unit	Anionic		[61]

[a] Degree of deacetylation depends on the source of chitosan and the process of deacetylation.

[b] http://www.solimide.eu/de/pharmapolymers/eudragit/quality/spezifikationen_neu.Par.0001.TRow.0006.TCell.0002.File.tmp/7.1.03_INFO7.3e_L100_S100_200409.pdf.

Table 3
Dissociation of bound proteins from Eudragit L-100 by different dissociating agents.

Dissociating agent	Concentration of the dissociating agent	Recovery of protein (%)		
		CcdB-F17P	malETrx	CD4D12
NaCl	0.2 M	5.3	4.0	6.1
	0.5 M	8.2	5.7	8.6
	1.0 M	14.5	8.2	12.0
Ethylene glycol	50% (v/v)	55.3	60.4	60.0
	70% (v/v)	92.0	90.8	88.2
	90% (v/v)	93.2	91.5	89.6

The experiments were carried out in triplicates and the difference in the individual readings was less than 5%.

of 6.1 and 6.7 for CcdB-F17P and M97K, respectively (Table 4), the protein will not have a high amount of charge at the refolding pH of 7.5. Hence, it is understandable that electrostatic bonds do not dominate in this interaction. Fig. 2B shows that when the binding of CcdB-F17P to the polymer was carried out in the presence of 70% ethylene glycol, no size increase was observed. This indicated: (1) size increase with time indeed reflects binding of the polymer with the protein, (2) the binding is largely due to hydrophobic interaction as ethylene glycol is known to inhibit hydrophobic interactions [55].

The concentration of Eudragit L-100 used in the refolding experiments was 0.2% (w/v). Fig. 3A shows that this indeed was the minimum concentration which bound the maximum (>90%) of the protein. Using higher concentration of Eudragit L-100 would not be more useful. Fig. 3B shows that under these conditions, the protein concentration for refolding experiments can be raised up to 1.50 mg mL^{-1}. Increasing protein concentration beyond this would mean that a significant percentage of protein would remain unbound to the polymer and would be unlikely to refold.

3.2.1. Characterization of refolded CcdB mutants

The fluorescence emission spectra of WT-CcdB and the mutants are given in Fig. 4A. As it was indicated during the polymer screening data, both mutants have the same λ_{max} (emission) of 340 nm as WT-CcdB and hence fluorescence data shows that these are in the correctly folded, native-like forms. When both refolded mutants are incubated in 6 M GdmCl, there was a red shift in the spectra to 358 nm, along with increase in the fluorescence intensity due to exposure of tryptophan residues from the quenched environment, similar to WT-CcdB [51].

The CD spectra of the refolded mutants along with WT-CcdB are given in Fig. 4B. The CD spectrum of WT-CcdB was similar to what has been reported earlier [13]. The spectra of the mutants are very similar to the spectra of WT-CcdB indicating that the refolded mutants have native like structure.

ANS binding measurements were also carried out on the affinity precipitation refolded CcdB-F17P and CcdB-M97K, and compared with the WT-CcdB (Fig. 5). WT-CcdB does not bind to ANS in its native state at pH 7.0 due to its low surface hydrophobicity and presence of a folded dimeric form at pH 7.0. However, ANS binding was observable at pH 4.0 in WT-CcdB because the dimer dissociates into folded monomers [51], increasing its surface hydrophobicity at pH 4.0. Similar behavior was seen in the cases of refolded CcdB-F17P and M97K mutants, indicating a folded dimeric CcdB at pH 7.0, whereas at pH 4.0, the CcdB dimer dissociates into folded monomers.

Fig. 6A shows the SDS-PAGE analysis of inclusion bodies (washed and unwashed) and refolded CcdB mutants. As seen in lanes 4 and 7, refolding of the CcdB mutants was accompanied by simultaneous purification. The refolded CcdB mutants were homogenous by SDS-PAGE analysis.

T_m of the refolded CcdB mutants along with WT-CcdB, were also determined by CD (Table 4). The refolded CcdB mutants had T_m very similar to WT-CcdB.

Table 4
Melting temperature (T_m) and other physicochemical properties of different proteins refolded by affinity precipitation and their comparison with soluble native (wild type) proteins.

Name of the protein	T_m of the protein (°C)[a]	Molecular weight (Da)[b]	Amino acids[b]	Isoelectric point[b]	Secondary structure content[c]
WT-CcdB	65.0	11,706	101	6.1	Alpha Helix: 25 Beta Sheet: 30
Refolded CcdB-F17P	61.0	11,656	101	6.1	Alpha Helix: 26 Beta Sheet: 28
Refolded CcdB-M97K	60.0	11,703	101	6.7	Alpha Helix: 26 Beta Sheet: 28
WT-Thioredoxin	84.0	11,675	108	4.7	Alpha Helix: 28 Beta Sheet: 23
Refolded malETrx	85.2	14,355	134	5.3	Alpha Helix: 29 Beta Sheet: 22
Refolded CD4D12	61.0	20,262	183	9.0	Alpha Helix: 14 Beta Sheet: 38
WT-MBP	64.0	40,707	370	5.2	Alpha Helix: 36 Beta Sheet: 18
Refolded MBP224D	63.0	40,691	370	5.1	Alpha Helix: 34 Beta Sheet: 17
Refolded MBP264D	62.0	40,735	370	5.1	Alpha Helix: 33 Beta Sheet: 18
Refolded ScFv b12	53.0	28,130	254	9.1	Alpha Helix: 15 Beta Sheet: 34
Refolded ScFab b12	59.0	51,014	479	8.8	Alpha Helix: 18 Beta Sheet: 31

[a] As determined by CD.
[b] Calculated by using ProtParam program (http://web.expasy.org/protparam/) [62].
[c] As calculated from CD spectra by using K2d software package [43].

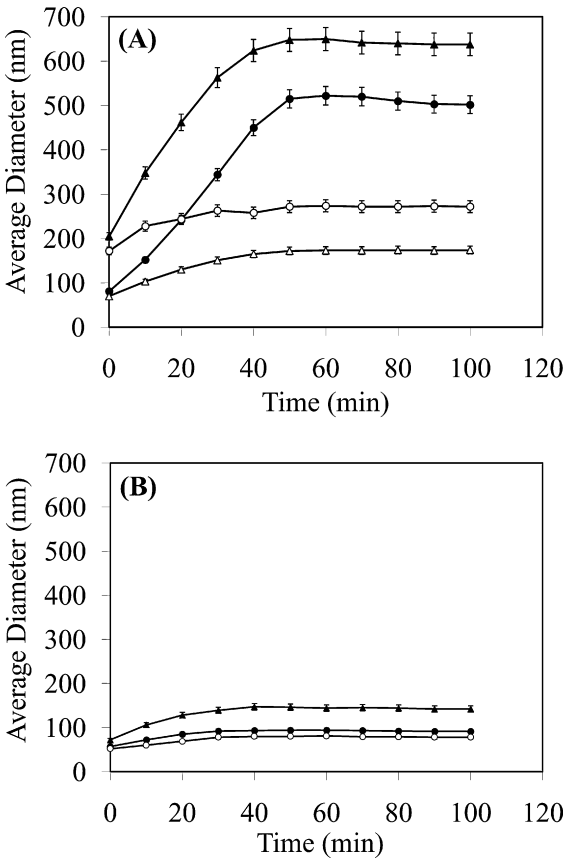


Fig. 2. The size distribution analysis to show the binding of the protein molecules with the smart polymers with time at 25 °C by dynamic light scattering experiment for: (A) Smart polymers, Eudragit L-100 with solubilized inclusion bodies of: (●) CcdB-F17P and (△) ScFv b12, and Protanal LF with solubilized inclusion bodies of: (○) CcdB-F17P and (▲) ScFv b12. (B) Eudragit L-100 with solubilized inclusion bodies of CcdB-F17P in the presence of: (▲) 20% ethylene glycol, (●) 50% ethylene glycol and (○) 70% ethylene glycol. In the above experiments the Eudragit L-100 concentration was 0.2% (w/v), Protanal LF concentration was 0.5% (w/v) and the protein concentration was 0.5 mg mL^{-1} in a total volume of 0.2 mL in 10 mM Tris–HCl buffer, pH 7.5 for CcdB F-17P and pH 7.0 for ScFv b12. The experiments were carried out in triplicates and the error bars represent the difference among the individual readings.

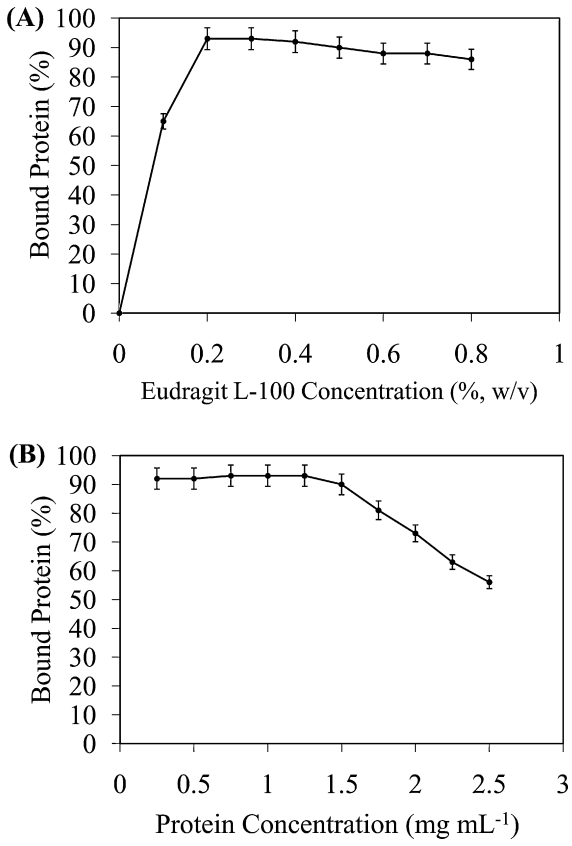


Fig. 3. (A) Binding of solubilized inclusion bodies of CcdB-F17P with Eudragit L-100 at different concentrations of Eudragit L-100 at 25 °C. The protein concentration was 0.5 mg mL^{-1} in a total volume of 0.2 ml in 50 mM Tris–HCl, pH 7.5. (B) Binding of solubilized inclusion bodies of CcdB-F17P with Eudragit L-100 at different protein concentrations at 25 °C. The Eudragit L-100 concentration was 0.2% (w/v) in a total volume of 2 mL in 50 mM Tris–HCl, pH 7.5. The experiments were carried out in triplicates and the error bars represent the difference among the individual readings.

3.3. Refolding of malETrx and human CD4D12 from inclusion bodies

The fusion protein of thioredoxin with signal peptide of MBP (malETrx) and human CD4D12, both could be similarly refolded from their inclusion bodies with Eudragit L-100. Thioredoxin is a small (108 amino acids in *E. coli*) protein which is present in a wide variety of organisms [56]. Thioredoxin has many functions mainly maintaining the redox state of target proteins by its active site containing an exposed disulfide/dithiol. For studying the effect of signal peptide on protein stability, a fusion protein of thioredoxin (malETrx) with the signal peptide of MBP was created, which formed inclusion bodies upon expression in *E. coli* and could not be refolded by conventional refolding techniques [13]. CD4D12 corresponds to the first two N-terminal domains (amino acids 1–183) of human CD4. CD4D12 has been shown to bind gp120 of HIV-1 and inhibit HIV-1 infection in vitro and is being used for research in therapeutics and vaccine development against HIV infection [32,57]. When the CD4D12 construct is expressed in *E. coli*, it forms inclusion bodies [13]. In these cases as well, both the properties of the proteins (Table 4) and Eudragit L-100 (Table 2) and the dissociation with 70% ethylene glycol reflected that the binding was again largely due to hydrophobic interactions (Table 3).

The intrinsic fluorescence emission spectra, ANS fluorescence spectra and CD spectra of the refolded malETrx are given in Fig. 7. The fluorescence spectra showed an emission λ_{max} at 342 nm, indicating the correctly folded native like conformation. Incubation of the refolded malETrx in 6 M GdmCl resulted in a red shift to 356 nm with increase in the fluorescence intensity. The identical nature of CD spectra of refolded malETrx with pure WT-thioredoxin further confirmed the successful refolding. ANS binding was not observed in both the cases of WT-thioredoxin and refolded malETrx. The refolded malETrx was assayed by the insulin aggregation assay for thioredoxin activity [44]. The specific activity of 3.3 of the refolded malETrx compared well with the known thioredoxin sample (Table 5). The refolded protein was homogenous on SDS-PAGE analysis (Fig. 6B).

Table 5
Insulin aggregation assay for thioredoxin proteins.

	Protein (mg)	Activity (Units)[a]	Specific-activity (Units mg^{-1})
Initial protein taken for refolding (solubilized inclusion bodies)	0.50 mg	–	–
Affinity precipitation refolded malETrx	0.32 mg	1.22	3.3
WT-Thioredoxin (Sigma–Aldrich)	–	–	4.0

The experiments were carried out in triplicates and the difference in the individual readings was less than 5%.

[a] One unit will cause a ΔA650 of 1.0 in 1 min at 25 °C.

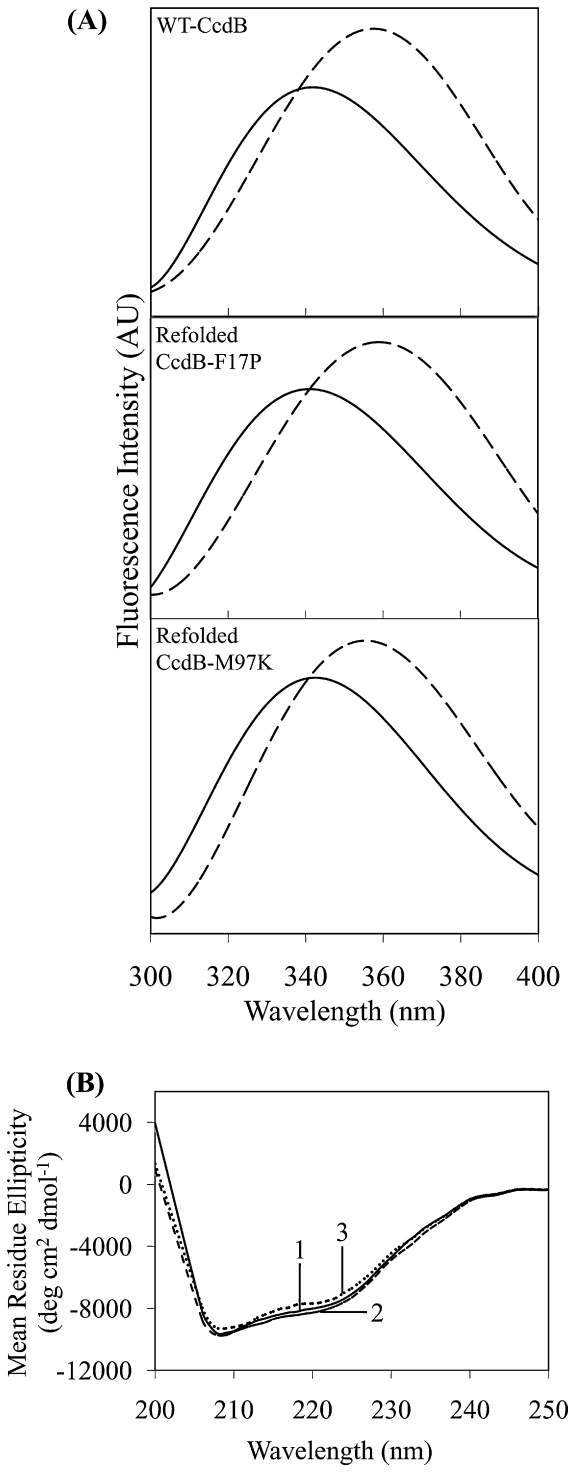


Fig. 4. (A) Fluorescence emission spectra (AU, arbitrary units) of CcdB proteins in 10 mM Tris–HCl, pH 7.5, with excitation at 280 nm using excitation and emission slit widths of 2 nm and 5 nm, respectively. Protein samples at a concentration of 1 μM were incubated either in Tris–HCl buffer (—); or in 6 M GdmCl (– –), 3 h before spectral acquisition. (B) Far-UV CD spectra of CcdB proteins with 15 μM protein concentration in 10 mM Tris–HCl, pH 7.5: (1) WT-CcdB, (2) refolded CcdB-F17P and (3) refolded CcdB-M97K.

Similarly, intrinsic fluorescence, ANS fluorescence and CD spectra of the refolded CD4D12 (Fig. 8) were similar to the CD4D12 purified by conventional methods [32]. Intrinsic fluorescence spectra showed an emission maximum of 341 nm while on incubation with 6 M GdmCl, the emission maximum was red shifted to 357 nm along with increase in the fluorescence intensity. ANS did not bind to the affinity precipitated and refolded CD4D12. The CD spectrum was characteristic of higher β-sheet content. Its T_m by CD was found out to be 61 °C (Table 4) and is quite close to T_m of CD4D12 refolded by dilution. Refolded CD4D12 was also simultaneously purified to homogeneity as shown by SDS-PAGE analysis (Fig. 6B).

3.4. Refolding of two mutants of MBP (224D and 264D) from inclusion bodies

MBP is a 370 amino acid periplasmic *E. coli* protein encoded by the malE gene and is involved in maltose binding and transport. MBP is a protein of wide interest to researchers [58]. Aspartate substitution at positions 224 and 264 in MBP resulted in the formation of inclusion bodies with inactive protein when expressed in *E. coli* [31]. Both the MBP mutants were refolded by precipitation with cationic starch using the protocol earlier reported for purification of soluble MBP and MBP fusion proteins [36]. Fig. 9A shows the increase in size with time when the cationic starch and solubilized inclusion bodies of MBP224D are incubated together. The binding did not take place if NaCl was present in the medium. It has been already pointed out in an earlier paper that MBP and MBP fusion proteins interact with cationic starch via electrostatic forces [36]. The absence of the increase in size in the presence of salt confirms that this size increase was in fact due to binding of the polymer with the protein. Maltose binding is known to cause a red shift along with quenching in the fluorescence spectra of MBP [45]. Fig. 10A shows that the refolded MBP mutants displayed the red shift and the quenching in the intrinsic fluorescence upon binding with maltose. The ANS binding was not observed either for WT-MBP or for both the MBP mutants refolded by affinity precipitation with cationic starch (Fig. 10B). T_m of refolded MBP mutants was close to WT-MBP (Table 4). The SDS-PAGE analysis of refolded MBP mutants which showed purified proteins is shown in Fig. 6C.

3.5. Refolding of two antibody fragments (ScFv b12 and ScFab b12) from inclusion bodies

Single chain variable fragment (ScFv) b12 and single chain antigen binding fragment (ScFab) b12 are both antibody fragments of different sizes derived from the anti-HIV-1 antibody b12 which broadly neutralizes HIV-1 [33]. These recombinant antibody fragments are of much significance for HIV research and developing anti-HIV therapeutics. When both of these antibody fragments are expressed in *E. coli*, they result in the formation of inclusion bodies and the resultant protein is inactive. Protanal LF (alginate with high guluronic acid content) was found to refold the two antibody fragments (ScFv b12 and ScFab b12) and the refolding yield was much higher than conventional refolding by dilution. The incorporation of NaCl in the buffer resulted in dissociation of the protein from the polymer. This is similar to the result with a lipase using the same alginate [29]. Protanal LF is an anionic polymer (Table 2) and both the antibody fragments ScFv b12 and ScFab b12 have isoelectric points of about 9.1 and 8.8, respectively, and are positively charged at refolding pH of 7.0 (Table 4). In view of this, it can be presumed that the binding with the protein is largely via electrostatic interactions. Also, the absence of increase in size when the polymer and the solubilized inclusion bodies of ScFv b12 were incubated in the presence of salt (NaCl) confirms that the size increase was due to actual binding between the polymer and the protein (Fig. 9B). ScFv b12 and ScFab b12 are recombinant forms of wild type antibody fragments which form inclusion bodies, so no comparison with any originally folded structure was possible. Fluorescence emission spectra showed a maximum at 340 nm for both the proteins, characteristic of native structure (Fig. 11A). Also the CD spectra showed secondary structure with high β-sheet content, characteristic of these antibody fragments (Fig. 11B) [59]. The T_m was also

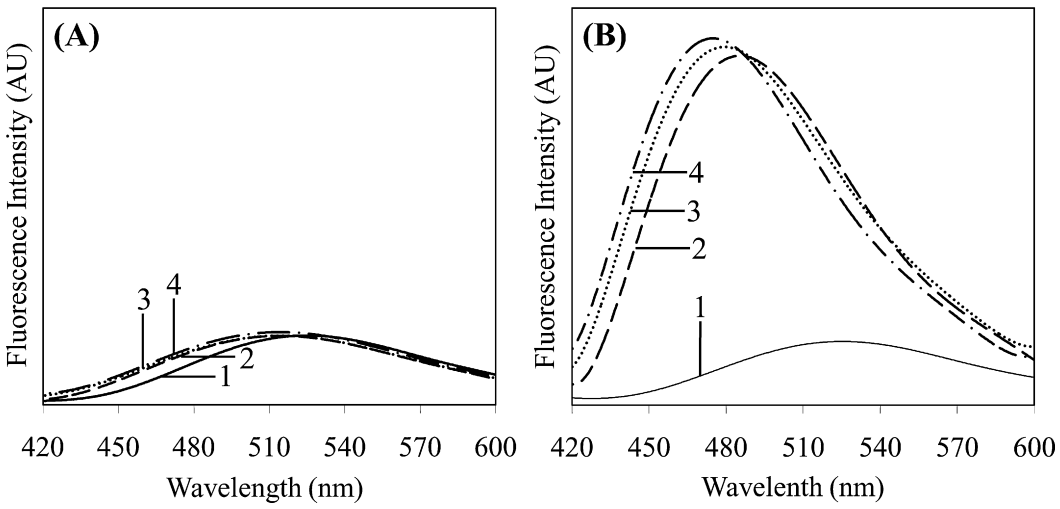


Fig. 5. ANS fluorescence emission spectra (AU, arbitrary units) of CcdB proteins with excitation at 390 nm using excitation and emission slit widths of 2 nm and 5 nm, respectively. The protein and ANS concentrations used were 1 and 100 μM, respectively. The samples were incubated for 30 min before spectral acquisition in: (A) 25 mM sodium phosphate buffer pH 7.0 and (B) 25 mM sodium acetate buffer pH 4.0. In both the figures, curves 1, 2, 3 and 4 represent ANS without protein, ANS with WT-CcdB, ANS with refolded CcdB-F17P and ANS with refolded CcdB-M97K, respectively.

Table 6
Refolding yields (mg L^{-1} of culture)[a] of proteins refolded by affinity precipitation with smart polymers.

S. no.	Protein	Yield by affinity precipitation (mg L^{-1} of culture)
1.	CcdB-F17P	32 ± 2
2.	CcdB-M97K	30 ± 2
3.	malETrx	58 ± 2
4.	CD4D12	8 ± 1
5.	MBP224D	40 ± 2
6.	MBP264D	43 ± 2
7.	ScFv b12	30 ± 2
8.	ScFab b12	26 ± 2

[a] Method for calculating yield (mg L^{-1} of culture): ([total purified protein {mg} × fraction of pure protein]/culture volume [mL]) × 1000.

measured by CD (Table 4). Both the refolded antibody fragments were also analyzed by SDS-PAGE (Fig. 6D).

3.6. Refolding yields

The refolding yields (mg L^{-1}) of the various proteins are reported in Table 6. As mentioned earlier [13], many of the proteins (i.e. CcdB and CD4D12) have been difficult to refold by conventional methods as these are prone to aggregation. It is seen that reasonable yields of refolded proteins could be obtained in all the cases.

3.7. Understanding the role of smart polymers in refolding

The results given above indicated that the smart polymers actually bind to the proteins and this step is responsible in some way for correct refolding of the proteins. In order to directly confirm that such binding indeed takes place, the polymers Eudragit L-100 and Protanal LF were incubated with CcdB-F17P and ScFv b12, respectively under the conditions of refolding. In both cases, the polymers were precipitated at different time intervals by appropriate stimuli (as described in Sections 2.9 and 2.11). Fig. 12A shows that proteins were also precipitated along with the polymers. The amount of protein precipitated (in both cases) increased with time and reached a plateau at around 50 min. In each case, the bound protein could be dissociated by appropriate materials (70% ethylene glycol for Eudragit L-100 and 1 M NaCl for Protanal LF) (Fig. 12B). This confirmed that the protein disappearing from the supernatant was in fact due to precipitation along with the polymer at each point in time. In case of CcdB-F17P, the proteins dissociated from Eudragit

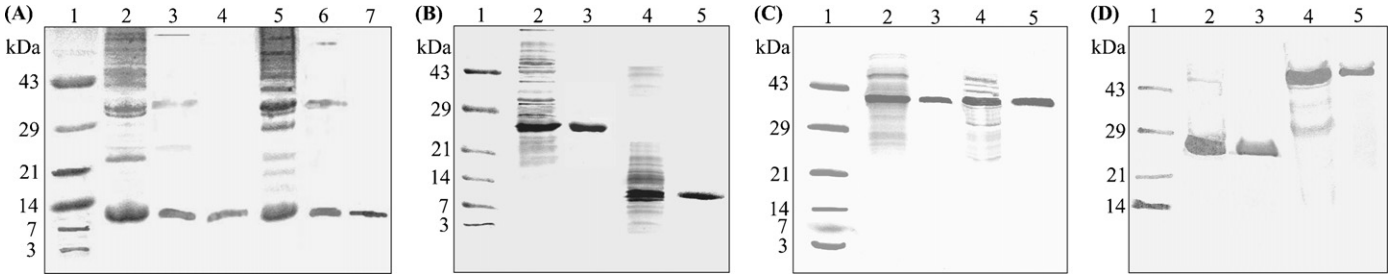


Fig. 6. SDS-PAGE analysis of the proteins refolded and purified by smart polymers from inclusion bodies. (A) Fifteen percent SDS-PAGE analysis showing purification of CcdB mutants with Eudragit L-100; (lane 1) molecular weight markers; (lane 2) unwashed inclusion bodies of CcdB-F17P; (lane 3) washed inclusion bodies of CcdB-F17P with 50 mM PBS/pH 7.4/0.5% Triton X-100; (lane 4) refolded and purified CcdB-F17P; (lane 5) unwashed inclusion bodies of CcdB-M97K; (lane 6) washed inclusion bodies of CcdB-M97K with 50 mM PBS/pH 7.4/0.5% Triton X-100; (lane 7) refolded and purified CcdB-M97K. (B) Fifteen percent SDS-PAGE analysis showing purification of CD4D12 and malETrx with Eudragit L-100; (lane 1) molecular weight markers; (lane 2) washed inclusion bodies of CD4D12 with 50 mM PBS/pH 7.4/0.5% Triton X-100; (lane 3) refolded and purified CD4D12; (lane 4) washed inclusion bodies of malETrx with 50 mM PBS/pH 7.4/0.5% Triton X-100; (lane 5) refolded and purified malETrx. (C) Fifteen percent SDS-PAGE analysis showing purification of MBP mutants with cationic starch; (lane 1) molecular weight markers; (lane 2) washed inclusion bodies of MBP224D with 50 mM PBS/pH 7.4/0.5% Triton X-100; (lane 3) refolded and purified MBP224D; (lane 4) washed inclusion bodies of MBP264D with 50 mM PBS/pH 7.4/0.5% Triton X-100; (lane 5) refolded and purified MBP264D. (D) Fifteen percent SDS-PAGE analysis showing purification of ScFv b12 and ScFab b12 with Protanal LF; (lane 1) molecular weight markers; (lane 2) washed inclusion bodies of ScFv b12 with 50 mM PBS/pH 7.4/0.5% Triton X-100; (lane 3) refolded and purified ScFv b12; (lane 4) washed inclusion bodies of ScFab b12 with 50 mM PBS/pH 7.4/0.5% Triton X-100; (lane 5) refolded and purified ScFab b12.

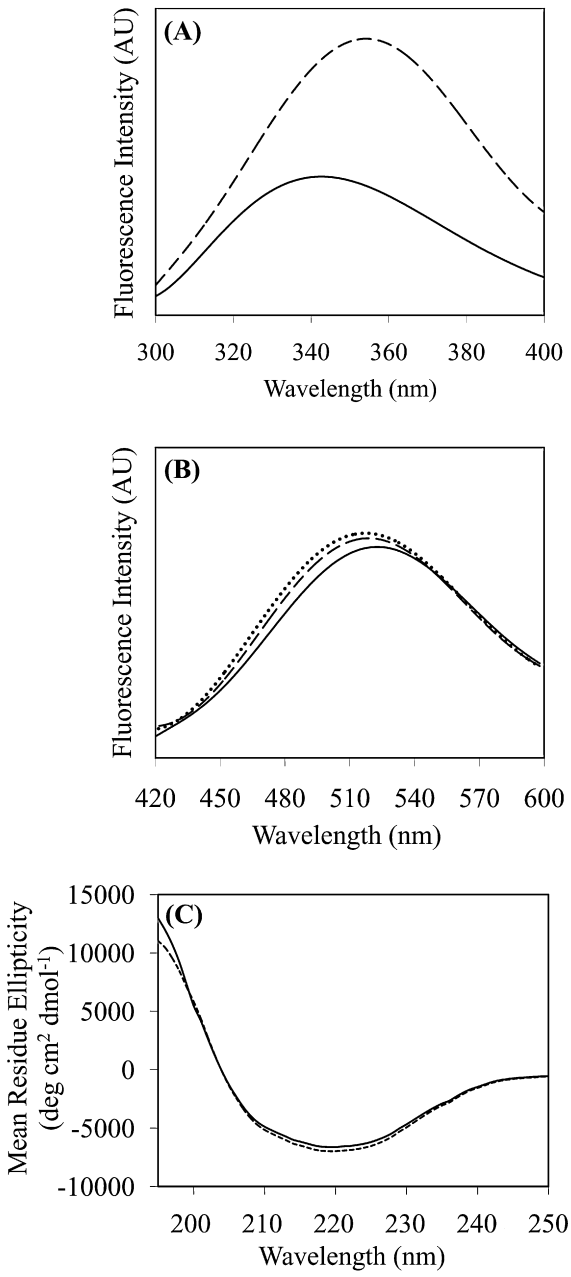


Fig. 7. (A) Fluorescence emission spectra (AU, arbitrary units) of thioredoxin proteins with 1 μM protein concentration in 10 mM Tris–HCl, pH 7.5, with excitation at 280 nm using excitation and emission slit widths of 2 nm and 5 nm, respectively: (—) refolded malETrx and (– –) unfolded malETrx in 6 M GdmCl. (B) ANS fluorescence emission spectra (AU, arbitrary units) of thioredoxin proteins with excitation at 390 nm using excitation and emission slit widths of 2 nm and 5 nm, respectively. The protein and ANS concentrations used were 1 and 100 μM, respectively, in 10 mM Tris–HCl, pH 7.5: (—) ANS without protein, (– –) ANS with WT-thioredoxin (Sigma–Aldrich) and (····) ANS with refolded malETrx. (C) Far-UV CD spectra of thioredoxin proteins with 15 μM protein concentration in 10 mM Tris–HCl, pH 7.5: (—) WT-thioredoxin (Sigma–Aldrich) and (- -) refolded malETrx.

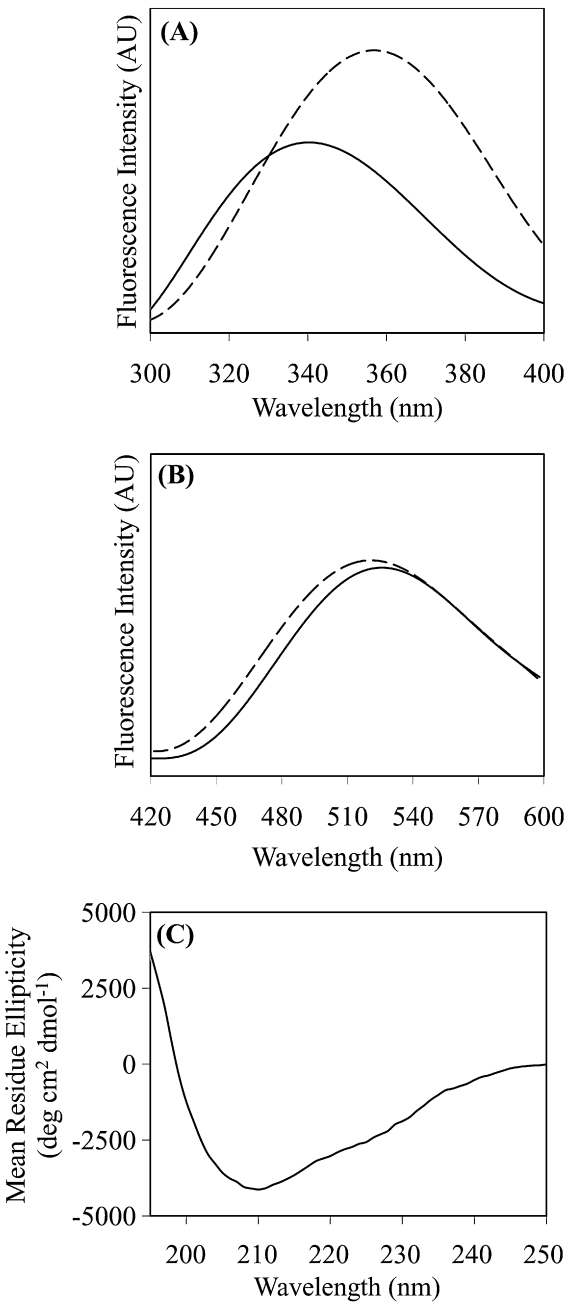


Fig. 8. (A) Fluorescence emission spectra (AU, arbitrary units) of CD4D12 with 1 μM protein concentration in 10 mM Tris–HCl, pH 7.5, with excitation at 280 nm using excitation and emission slit widths of 2 nm and 5 nm, respectively: (—) refolded CD4D12 and (– –) unfolded CD4D12 in 6 M GdmCl. (B) ANS fluorescence emission spectra (AU, arbitrary units) of CD4D12 with excitation at 390 nm using excitation and emission slit widths of 2 nm and 5 nm, respectively. The protein and ANS concentrations used were 1 and 100 μM, respectively, in 10 mM Tris–HCl, pH 7.5: (—) ANS without protein and (– –) ANS with refolded CD4D12. (C) Far-UV CD spectra of CD4D12 with 15 μM protein concentration in 10 mM Tris–HCl, pH 7.5.

L-100 at each point in time were characterized by CD (Fig. 12C and Table 7). It is interesting to note that whatever protein was bound to the polymer (and dissociated thereafter) at any time was similar in secondary structure content as wild type native protein. On the other hand, the protein remaining in the supernatant (which did not bind to the polymer) was found to have significant amount of random coil structure (Supplementary Table S1). This confirmed that the protein binding to the smart polymer was necessary for its refolding. This data along with data in Figs. 2, 9 and 12, strongly suggests that protein refolding was not a rate limiting step and was very fast. This is in line with current understanding of protein folding/refolding process [5]. Hence the time period of about 50 min required for refolding the proteins was due to the slow binding kinetics of the proteins with the smart polymers. The identical kinetic data with various systems (involving different smart polymers and different proteins) supports the possibility that there is a common pathway in all the cases.

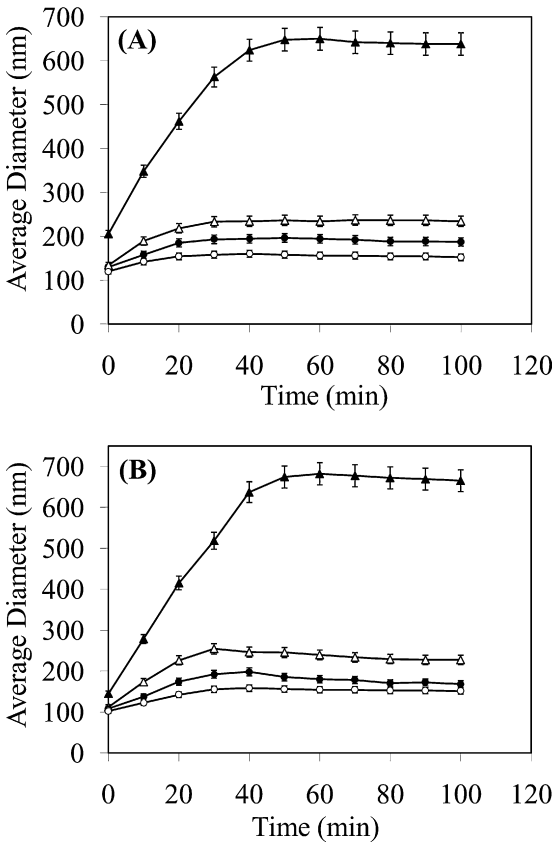


Fig. 9. The size distribution analysis to show the binding of the protein molecules with the smart polymers with time at 25 °C by dynamic light scattering experiment for: (A) MBP 224D with cationic starch in the presence of: (▲) Buffer (50 mM Tris–HCl pH 7.5), (△) 0.5 M NaCl in 50 mM Tris–HCl pH 7.5, (●) 0.5 M NaCl in 50 mM Tris–HCl pH 7.5 and (○) 1 M NaCl in 50 mM Tris–HCl pH 7.5. (B) ScFv b12 with Protanal LF in the presence of: (▲) Buffer (50 mM Tris–HCl pH 7.0), (△) 0.2 M NaCl in 50 mM Tris–HCl pH 7.0, (●) 0.5 M NaCl in 50 mM Tris–HCl pH 7.0 and (○) 1 M NaCl in 50 mM Tris–HCl pH 7.0. In the above experiments the cationic starch concentration was 0.3% (w/v), Protanal LF concentration was 0.5% (w/v) and the protein concentration was 0.5 mg mL^{-1} in a total volume of 2 mL. The experiments were carried out in triplicates and the error bars represent the difference among the individual readings.

Table 7
Secondary structure analysis of the CcdB-F17P recovered from the Eudragit L-100 after incubation with varying time intervals.

S. no.	Incubation time with Eudragit L-100 before precipitation (min)	Secondary structure content of the dissociated protein from precipitated Eudragit L-100 (%)
1.	5	α-Helix: 25 β-Sheet: 26 Random coil: 49
2.	10	α-Helix: 26 β-Sheet: 25 Random coil: 49
3.	20	α-Helix: 27 β-Sheet: 25 Random coil: 48
4.	30	α-Helix: 26 β-Sheet: 27 Random coil: 47
5.	40	α-Helix: 26 β-Sheet: 28 Random coil: 46
6.	50	α-Helix: 26 β-Sheet: 29 Random coil: 45
7.	60	α-Helix: 26 β-Sheet: 29 Random coil: 45

The secondary structure content of WT-CcdB is: α-helix: 25, β-sheet: 30 random coil: 45 and denatured CcdB-F17P is: α-helix: 10, β-sheet: 25, random coil: 65.

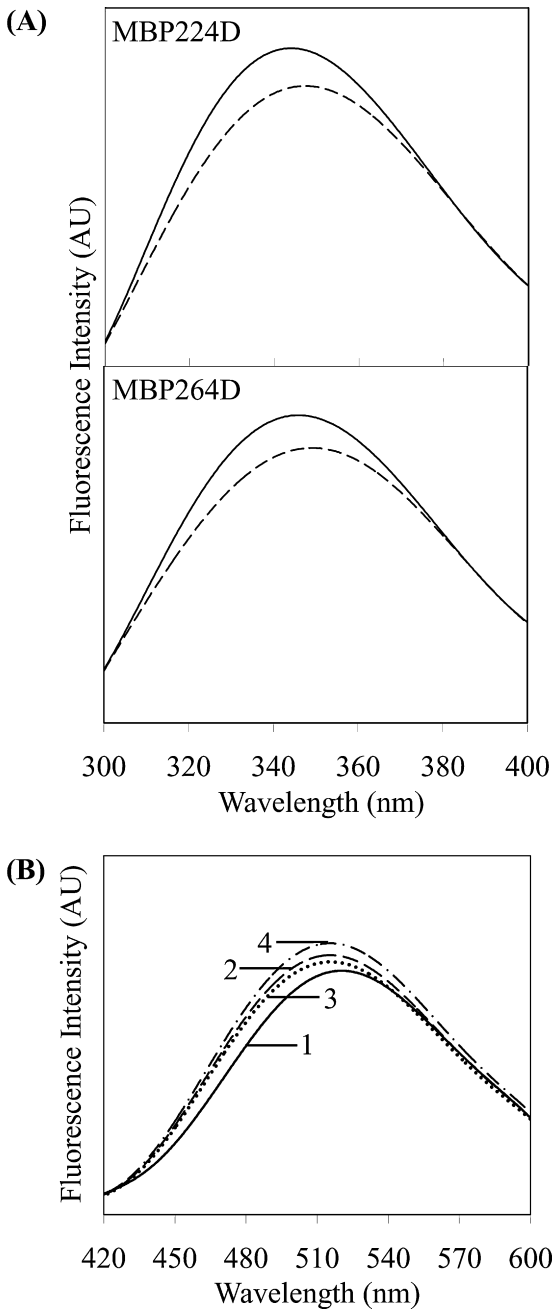


Fig. 10. (A) Fluorescence based assay of refolded MBP mutants. Fluorescence emission spectra (AU, arbitrary units) were acquired in 10 mM Tris–HCl, pH 7.5, with excitation at 280 nm using excitation and emission slit widths of 2 nm and 5 nm, respectively. Protein samples at a concentration of 3.5 μg mL^{-1} were incubated either in Tris–HCl buffer (—); or in Tris–HCl buffer containing 0.1 mM maltose (– –), 15 min before spectral acquisition. (B) ANS fluorescence emission spectra (AU, arbitrary units) of MBP proteins with excitation at 390 nm using excitation and emission slit widths of 2 nm and 5 nm, respectively. The protein and ANS concentrations used were 1 and 100 μM, respectively, in 10 mM Tris–HCl, pH 7.5. In both the figures, curves 1, 2, 3 and 4 represent ANS without protein, ANS with WT-MBP, ANS with refolded MBP224D and ANS with refolded MBP264D, respectively.

To sum up, it appears that the underlying mechanism of the refolding with these smart polymers is similar to the one described in the case of PEG facilitated refolding of guanidine hydrochloride denatured carbonic anhydrase B [15]. Unlike PEG, smart polymers seem to function very efficiently and facilitate simultaneous recovery and purification of the refolded protein.

4. Discussion

Based upon the above results, it appears that it is possible to select a suitable smart polymer which binds adequately to a given protein. The information about the nature of binding between the specific polymer and the protein being refolded can be inferred from the following kinds of data. For CcdB mutants, Eudragit L-100 was identified as the appropriate smart polymer (Table 1). Eudragit L-100 has more charges than Eudragit S-100 as the latter has a larger percentage of carboxylate groups present in the esterified form (Table 2). According to the screen (Table 1), both polymers refolded CcdB mutants. We had reported earlier that Eudragit S-100 does refold a CcdB mutant [19] but the protein could not be dissociated from the polymer. With Eudragit L-100, dissociation with ethylene glycol works. So, the binding seems to be predominantly through hydrophobic forces. In view of larger content of the ester groups in Eudragit S-100, this interaction is expected to be stronger so it is understandable that the CcdB mutant could not be dissociated from Eudragit S-100. Furthermore, Fig. 2B based upon the DLS data showed that the binding (as reflected by increasing size) did not take place between Eudragit L-100 and CcdB-F17P in the presence of ethylene glycol. In cases of CD4D12 and malETrx refolding the optimal smart polymer again is Eudragit L-100 and the refolded protein is dissociated by ethylene glycol. So, a similar hydrophobic nature of binding can be presumed in these cases as well.

The two MBP mutants have been refolded by cationic starch and dissociated from the polymer by NaCl. Given the structure of the polymer and dissociating buffer, it can be inferred that the nature of binding is predominantly via ionic bonds. The MBP mutants have an isoelectric point of 5.1 (Table 4), hence the proteins are expected to be negatively charged at the refolding pH of 7.5. Hence considerable interaction with cationic starch (Table 2) would be expected.

The two antibody fragments ScFv b12 and ScFab b12 are refolded by Protanal LF and dissociated by NaCl. Protanal LF is an alginate (Table 2) with many free carboxylate groups and again it looks very likely that the interaction between the polymer and the proteins is largely ionic in nature.

The smart polymers work as pseudochaperonins and push the protein down the correct refolding pathway as discussed earlier [9,10,12]. It is likely that binding by the polymer prevents aggregation which is known to compete with refolding. The general picture has been that such pseudochaperonins interact with hydrophobic sites on the unfolded protein molecule and prevent aggregation. In the present instances, some of the polymers (e.g. alginate) are fairly polar. It is likely that even in such cases, irrespective of the nature of the primary interaction; such polymers block interprotein interaction and consequently aggregation. This is not an unrealistic picture if one remembers that hydrophobic sites are interspersed in a protein chain and are surrounded by stretches of polar residues. Considering that a large number of smart polymers (both synthetic and natural ones) are known, the results of this study promise that a suitable 'pseudochaperonin' for any protein perhaps can be identified. Hence, the method outlined here may be widely applicable.

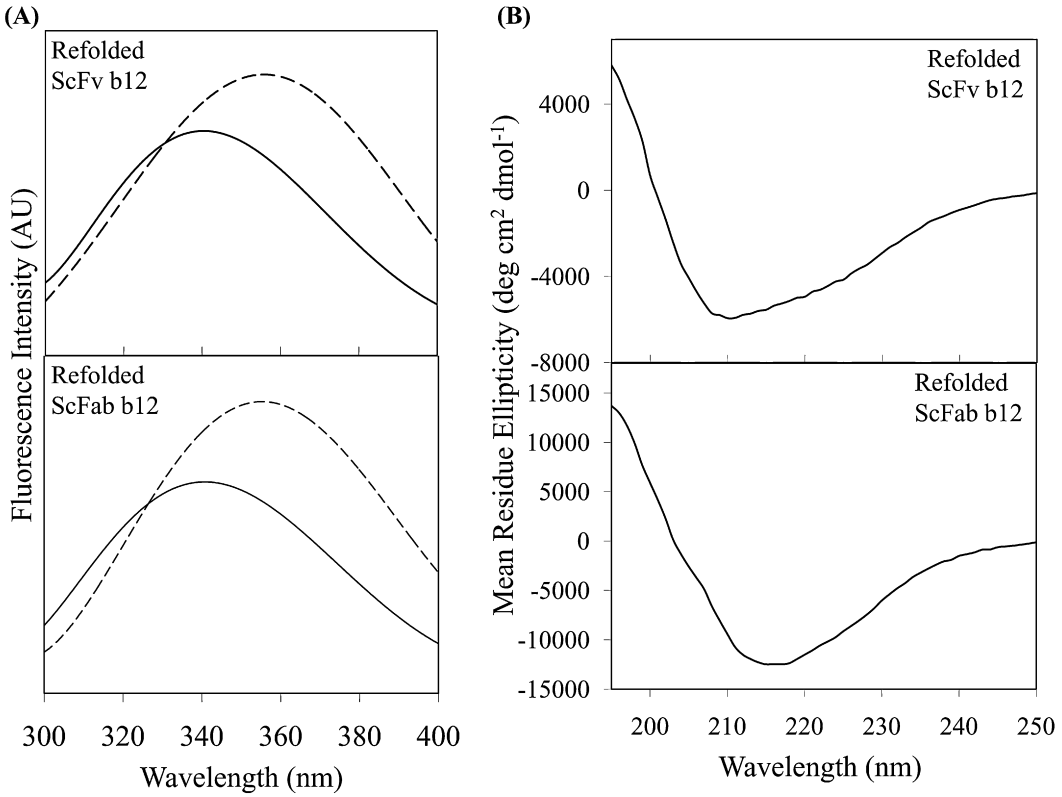


Fig. 11. (A) Fluorescence emission spectra (AU, arbitrary units) of refolded ScFv b12 and ScFab with 1 μM protein concentration in 10 mM Tris–HCl, pH 7.0, with excitation at 280 nm using excitation and emission slit widths of 2 nm and 5 nm, respectively: (—) refolded protein and (– –) unfolded protein in 6 M GdmCl. (B) Far-UV CD spectra of ScFv b12 and ScFab b12 with 15 μM protein concentration in 10 mM Tris–HCl, pH 7.0.

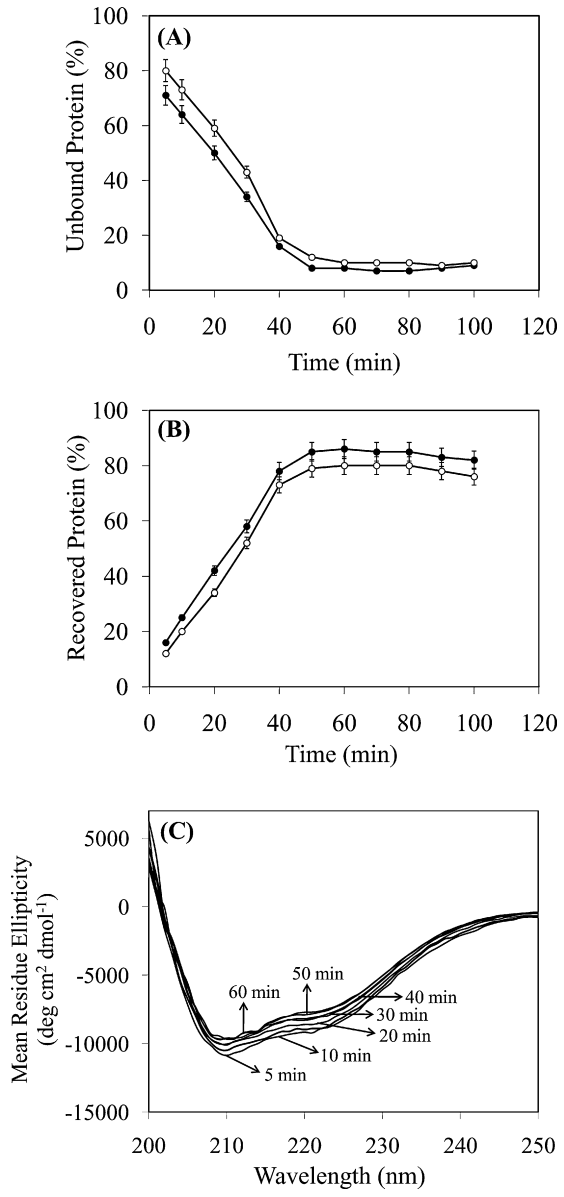


Fig. 12. (A) Unbound protein remaining in the supernatant after incubation of the smart polymers for different time intervals with the solubilized inclusion bodies of: (●) CcdB-F17P with Eudragit L-100 and (○) ScFv b12 with Protanal LF. (B) Effect of incubation time of the protein with the smart polymer on the recovery of proteins (after dissociation of the proteins by 70% ethylene glycol in case of CcdB-F17P and 1 M NaCl in case of ScFv b12, from the precipitated polymer–protein complex at varying time intervals): (●) CcdB-F17P from Eudragit L-100 and (○) ScFv b12 from Protanal LF. In the above experiments the Eudragit L-100 concentration was 0.2% (w/v), Protanal LF concentration was 0.5% (w/v) and the protein concentration was 0.5 mg mL^{-1} in a total volume of 2 mL in 50 mM Tris–HCl buffer, pH 7.5 for CcdB F-17P and pH 7.0 for ScFv b12. The experiments were carried out in triplicates and the error bars represent the difference among the individual readings. (C) Far-UV CD spectra of recovered CcdB-F17P from Eudragit L-100 at different time intervals in 10 mM Tris–HCl, pH 7.0. The Eudragit L-100 concentration was 0.2% (w/v) and the protein concentration was 0.5 mg mL^{-1} in a total volume of 0.2 mL for binding with varying time intervals. The protein was dissociated from Eudragit L-100 by 70% ethylene glycol. Binding and dissociation was carried out as described in Section 2.9.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2012.02.048.

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