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# Protein Refolding Assisted by Molecular Tube Based α-Cyclodextrin as an Artificial Chaperone

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**Abstract**—In this study, we evaluated, for the first time, the application of molecular tube based α-cyclodextrin for improving the refolding yields of two different enzymes: carbonic anhydrase and alkaline phosphatase. Our results indicate that under the optimal developed refolding environments, the denatured carbonic anhydrase and alkaline phosphatase were refolded with a yield of 51 and 61% using 15 and 5 mg/ml of the molecular tube, respectively. Regardless of lower refolding yields compared with liquid-phase artificial chaperone assisted approach, the new technique (solid-phase artificial chaperone assisted refolding) benefits from easier and faster separation of the refolded product from the refolding environment, recycling of the stripping agent, and finally, significantly less environmental effect at the industrial levels. However, further improvements in solid-phase artificial chaperone assisted technique are needed either through synthesizing better stripping agents or by optimizing and defining better refolding environments.

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*Key words*: alkaline phosphatase, artificial chaperone, carbonic anhydrase, α-cyclodextrin, molecular tube

Recombinant expression of exogenous proteins usually results in high expression levels of proteins. Unfortunately, most of these products form micron-scale intracellular inclusion bodies [1]. Inclusion bodies can be solubilized in concentrated chaotropes, and the denatured proteins can then be refolded *in vitro*, most conveniently by dilution in batch or fed-batch approaches. Protein aggregation often limits the refolding yield attainable when this convenient refolding route is employed [2]. Chaperone proteins or "chaperonins" are used to improve the refolding yields, based on their *in vivo* role of protecting intracellular proteins from aggregation. The use of chaperonins in bioprocesses is very costly and is limited by the stability of the chaperonins. Therefore, several other compounds have been evaluated for their ability to assist protein folding. L-Arginine [3-6] and polyethylene glycol (PEG) [7-9] are probably the most important and widely used additives, but denaturants such as guanidine hydrochloride, urea, and other detergents have also been found to significantly improve the refolding yield when applied in non-denaturing concentrations [10, 11]. The mechanism of how these additives assist protein refolding is still unclear. Cyclodextrins (CDs) have previously been used as additives to enhance the refolding yield of bovine carbonic anhydrase [12, 13] and α-amylase [14, 15]. It has been suggested that cyclodextrins form reversible complexes with the hydrophobic patches present on denatured proteins, thus preventing their aggregation without interfering with protein refolding [12]. In still another approach, higher refolding yields are obtained by the elegant approach of artificial chaperone developed by Rozema et al. [16-19]. In this method, the denatured protein solution is first diluted with a detergent solution followed by a second dilution step using a cyclodextrin solution. It is believed that the first detergent-based dilution step results in the protein being captured in mixed micelles. Cyclodextrin strips the detergent away from the non-native polypeptide chain, allowing complete refolding to the native structure. The method has been successfully applied for the

*Abbreviations*: ALP) alkaline phosphatase; ANS) 1-anilinonaphthalene-8-sulfonate; CA) bovine carbonic anhydrase; CD) cyclodextrin; CTAB) cetyltrimethylammonium bromide; CTAHS) cetyltrimethylammonium hydrogen sulfate; DTAB) dodecyltrimethylammonium bromide; GuHCl) guanidine hydrochloride; HMDI) hexamethylenediisocyanate; PEG) polyethylene glycol; pNPAc) *p*-nitrophenyl acetate; pNPP) *p*-nitrophenyl phosphate; TTAB) tetradecyltrimethylammonium bromide.

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refolding of lysozyme, bovine carbonic anhydrase, and citrate synthase [16-19]. Although independent refolding of several structurally diverse proteins by this approach have provided promising results regarding significant suppression of aggregation along with enhanced refolding yields, from the industrial point of view some modifications seem to be required to make the technique more economically efficient. In the liquid-phase artificial chaperone-assisted technique, the detergent and the free CD in the refolding solution should be removed from the refolded product and the discharge of the used CD solution into the environment would be problematic [20]. Immobilization of CDs on solid supports or their conversion to insoluble polymeric derivatives has been suggested as alternative ways of getting around the problems. By this strategy, the recycling of CDs will be feasible and the discharge into the environment would be significantly minimized to non-harmful levels. Various methods for insoluble CD production and/or immobilization on solid supports have been successfully developed and used [21]. However, these modifications may slow down their rates of detergent stripping and therefore leading to misfolding, aggregation, and lower refolding yields. To prevent or to weaken the extent of aggregation and/or misfolding, one approach would be to increase the number of cyclodextrin molecules per unit volume or mass of the solid matrix. In the 1990s, Harada et al. prepared a polyrotaxane in which many CD molecules were threaded onto a linear polymeric chain of polyethylene oxide (PEO) capped at both ends with bulky blocking groups [22]. Moreover, they successfully prepared a tubular conjugate called "molecular tube" by cross-linking adjacent hydroxyl groups of α-CDs in polyrotaxane [23, 24] and/or polypseudorotaxane [25]. In this study, we attempted to evaluate the refolding behaviors of denatured alkaline phosphatase (ALP) (a dimeric protein) and carbonic anhydrase (CA) (a monomeric protein) enzymes using both soluble α-CD and the insoluble α-CD molecular tube polymer as the stripping agents. Our results clearly indicated that, regardless of solid-phase artificial chaperone assisted benefits, the refolding yields are somewhat lower than the corresponding values obtained through liquid-phase artificial chaperone assisted method, meaning that further technical elaborations are required to optimize the new approach.

## MATERIALS AND METHODS

**Materials and equipment.** Alkaline phosphatase from calf intestine (ALP), bovine carbonic anhydrase (CA), *p*-nitrophenyl acetate (pNPAc), *p*-nitrophenyl phosphate (pNPP), guanidine hydrochloride (GuHCl), Tween 80, and Triton X-100 were purchased from Sigma (USA). Electrophoresis grade Tris base, cetyltrimethylammonium bromide (CTAB), cetyltrimethylammonium hydrogen sulfate (CTAHS), 1-anilinonaphthalene-8-sulfonate (ANS), tetradecyltrimethylammonium bromide (TTAB), and dodecyltrimethylammonium bromide (DTAB) were obtained from Merck (Germany). α-Cyclodextrin (α-CD) was from Acros Organic (Belgium). All other materials were of analytical grade and were used as obtained from the suppliers. Fluorescence measurements were performed in the ratio mode using a Varian spectrofluorometer, model Cary Eclipse, equipped with a xenon lamp. For monitoring the changes in secondary structure of proteins, an AVIV spectropolarimeter model 215 (USA) was used. A Cary-100 Bio Varian (Australia) UV-Vis spectrophotometer was used for turbidimetric analyses and protein determinations.

**Production of the molecular tube based α-cyclodextrin.** Insoluble molecular tube containing α-CD was prepared by polymerization of α-CD with hexamethylenediisocyanate (HMDI) as cross-linking agent in N,N-dimethylformamide solvent according to the published methods [22-25].

**Denaturation and refolding of ALP.** Denatured ALP was prepared by incubation of 2.4 mg/ml of the enzyme in 100 mM Tris-HCl buffer, pH 7.0, containing 3 M GuHCl at room temperature for 2 h. Protein denaturation was confirmed by activity determination as well as fluorescence measurement [26]. The ALP was then diluted by a factor of 15 with Tris-HCl buffer containing CTAB to give concentration of 0.16 mg/ml ALP (100 mM Tris-HCl, pH 7.0, 1 mM CTAB). After 10 min, α-CD stock solution (16 mM) and/or suspensions of various pre-weighed amounts of molecular tube were added to bring the final concentrations to 0.12 mg/ml ALP, 100 mM Tris-HCl, pH 7.0, 0.8 mM CTAB, and 4.8 mM α-CD, and/or various concentrations of molecular tube (5-20 mg/ml). After 3 h end-to-end gentle rocking at room temperature, the solid absorbent was removed by centrifugation and the supernatant was assayed for its enzymatic activity and also for its fluorescence and far-UV circular dichroism criteria. For refolding under dilution additive mode, the denatured ALP was directly refolded in refolding buffer containing 0.8 mM CTAB, 4.8 mM α-CD, and/or the molecular tube (5-20 mg/ml). After 3 h end-to-end gentle rocking at room temperature, the enzyme activities of the samples were measured.

**Denaturation and refolding of CA.** The enzyme (3 mg/ml) was denatured in 20 mM Tris-sulfate buffer, pH 7.75, containing 6 M GuHCl for 16 h at room temperature. Protein denaturation was confirmed by activity determination as well as fluorescence measurement. The mixture was diluted rapidly by 20 mM Tris-sulfate buffer containing detergent to give concentration of 0.043 mg/ml CA (20 mM Tris-sulfate buffer, pH 7.75, 2 mM CTAB). After incubation for 10 min, aliquots containing CA–detergent complexes were added to 16 mM α-CD stock solution and/or molecular tube suspension in water to bring the final concentrations to 0.03 mg/ml

CA, 20 mM Tris-sulfate, pH 7.75, 1.4 mM CTAB, 4.8 mM α-CD, and/or 5-20 mg/ml molecular tube. After overnight end-to-end gentle rocking at room temperature, the solid absorbent was removed by centrifugation and the supernatant was assayed for enzymatic activity, fluorescence, and far-UV circular dichroism criteria. For refolding under the dilution additive mode, the denatured CA was directly refolded in refolding buffer containing 1.4 mM CTAB, 4.8 mM α-CD, and/or the molecular tube (5-20 mg/ml). After overnight end-to-end gentle rocking at room temperature, the enzyme activities of the samples were measured.

**Enzyme assay.** The activity of ALP was determined based on pNPP hydrolysis, and the release of *p*-nitrophenolate anions was monitored at 405 nm [27]. The enzymatic activity of the renatured CA solution was determined based on pNPAc esterase activity of the enzyme according to Pocker and coworkers [28]. Protein concentrations were determined according to Lowry's method [29] using BSA as the standard, and the turbidity of each refolded sample was estimated by recording the absorbance at 400 nm.

**Circular dichroism measurement.** Changes in the secondary structure of the protein samples upon refolding were monitored in far-UV region (200-260 nm) using 1 mm path length cells and an AVIV spectropolarimeter model 215 (USA).

**Fluorescence measurements**. Fluorescence analyses were performed using a Varian spectrofluorometer, model Cary Eclipse, according to procedures described above. Excitation and emission slits were set at 5 nm.

**Residual CTAB measurement.** The residual CTAB concentration in the renaturation buffer after completion of the refolding process was measured based on ANS fluorescence according to Mennen et al. [20]. The refolded samples in the solid-phase artificial chaperone assisted refolding system were incubated with 100 μM ANS (final), and the fluorescence emission was measured at 480 nm after 30 min incubation at room temperature using 360 nm excitation wavelength with 1 cm path length quartz cell. Fluorescence intensity was plotted versus the final detergent concentration and the percent of residual detergent measured according to fluorescence intensity of each refolded sample.

## RESULTS AND DISCUSSION

**Synthesis of α-CD polymer.** The polymerization of α-CD molecules with the assistance of hexamethylenediisocyanate (HMDI) was confirmed by IR analysis. Figure 1a shows the IR spectrum of the molecular tube of α-CD and Figs. 1b and 1c represent the IR spectra of α-CD and HMDI, respectively. The absence of a peak at 2100 $cm^{-1}$ (related to isocyanate group) in Figs. 1a and 1b, in contrast to Fig. 1c, confirms the formation of the polymer.

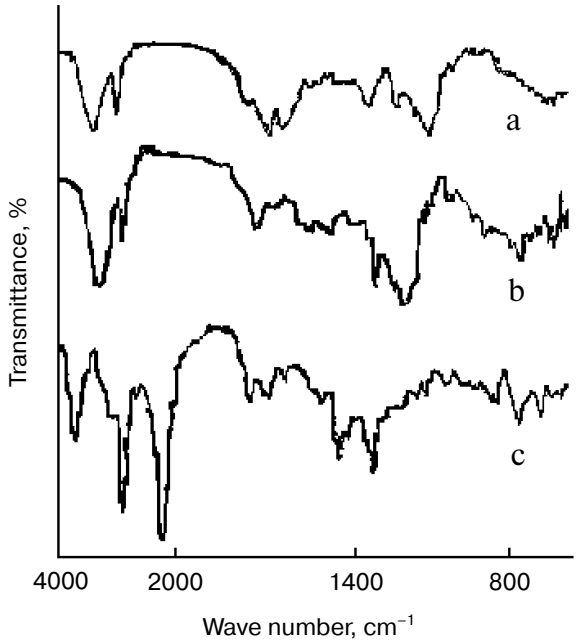


**Fig. 1.** Infrared (IR) spectra of (a) molecular tube, (b) α-cyclodextrin, and (c) HMDI.

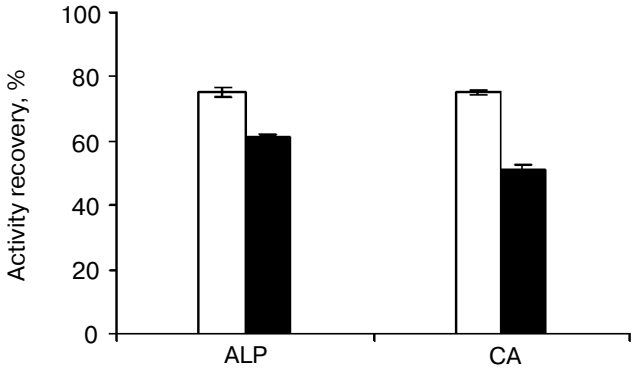


**Fig. 2.** Recovery of activity of denatured ALP and CA in the presence of α-CD (light columns) and the molecular tube (dark columns) in artificial-chaperone assisted refolding approach.

The peaks at 3370, 1720, and 1500 $cm^{-1}$ correspond to NH, CO, and NHCO groups, respectively. Based on Fig. 1a it is evident that the molecular tube between α-CD molecules is formed.

**Artificial chaperone assisted refolding of denatured ALP and CA.** The appropriate experimental conditions for refolding of GuHCl-denatured ALP and CA under the liquid-phase artificial chaperone assisted mode have already been worked out and published [13, 30]. Under these developed conditions, we were able to refold ALP and CA by almost 75%. In this investigation, we reevaluated the refolding of the same denatured proteins under the established refolding environment using the insoluble

**Table 1.** Recovery of activities of GuHCl-denatured ALP and CA in the presence of different kinds of ionic and non-ionic detergents in solid-phase artificial chaperone approach

| Additives | | Activity recovery, % | | | |
|---|---|---|---|---|---|
| | | Molecular tube, mg/ml | | | |
| | | 5 | 10 | 15 | 20 |
| ALP | CTAB (1 mM) | 61 ± 1.2* | 51 ± 2.2 | 46 ± 1.5 | 36 ± 2.7 |
| | TTAB (1 mM) | 52 ± 1.6 | 48 ± 3.0 | 40 ± 2.2 | 30 ± 1.9 |
| | DTAB (1 mM) | 44 ± 1.2 | 40 ± 0.7 | 35 ± 1.2 | 24 ± 2.2 |
| | Tween-80 (1 mM) | 32 ± 1.6 | 27 ± 1.2 | 25 ± 2.2 | 20 ± 1.5 |
| | Triton X-100 (1 mM) | 18 ± 2.2** | 13 ± 1.5 | 10 ± 1.9 | 4 ± 2.3 |
| CA | CTAB (2 mM) | 20 ± 1.2 | 38 ± 2.2 | 52 ± 1.5* | 48 ± 2.2 |
| | TTAB (2 mM) | 30 ± 3.0 | 38 ± 1.6 | 43 ± 2.5 | 40 ± 1.2 |
| | DTAB (2 mM) | 20 ± 1.9 | 22 ± 1.6 | 32 ± 1.2 | 32 ± 2.4 |
| | Tween-80 (2 mM) | 8 ± 1.2 | 8 ± 3.0 | 14 ± 2.4 | 12 ± 2.1 |
| | Triton X-100 (2 mM) | 9 ± 1.3 | 14 ± 1.6 | 15 ± 1.6** | 12 ± 2.4 |

Note: Each value represents the average of three independent measurements ± SD. Difference between values * vs. ** was statistically significant ($p < 0.001$).

molecular tube in place of soluble α-CD for the stripping step of the technique. As shown in Table 1, maximum refolding yield of 61% was achieved for GuHCl-denatured ALP with CTAB detergent, as the capturing agent, and 5 mg of α-CD polymer, as the stripping reagent. However, maximum refolding of GuHCl-denatured CA was achieved at 2 mM CTAB and 15 mg of the molecular tube of α-CD as the capturing and stripping agents, respectively. According to data presented in Table 1, the refolding recovery yields for both the cases of ALP and CA obey the order CTAB > TTAB > DTAB > Tween 80 > Triton X-100 as the capturing detergents. This is probably due to different affinities of the molecular tube for individual detergents, similar to the proven affinities of soluble α-CD for the detergents, which obey the same aforementioned order [30, 31]. Using the developed optimal refolding environment along with 4.8 mM of soluble α-CD and/or 5 mg of molecular tube per ml of the refolding medium, denatured ALP was refolded by about 75 and 61% under liquid- and solid-phase artificial chaperone assisted techniques, respectively (Fig. 2). Similarly, using 4.8 mM α-CD and/or 15 mg/ml molecular tube of α-CD the refolding yield was lower (51%) in solid-phase artificial chaperone assisted technique for CA compared to liquid-phase artificial chaperone assisted mode (75%). Lower refolding yields for both denatured enzymes were also obtained by the molecular tube at higher CTAB concentrations. These data rule out the possibility of inadequate protein capturing due to limiting amount of CTAB (Fig. 3). Additionally and according to Fig. 3, lower refolding yields for ALP and CA were obtained at molecular tube concentrations higher than the optimal values of 5 and 15 mg/ml, respectively. The decrease in refolding efficiency at higher than optimal concentrations of the molecular tube is possibly due to nonspecific binding of the refolded enzymes to the polymer. This possibility was ruled out by evaluating the absorption of native ALP and CA molecules to the resin under the refolding environments. No absorptions to the resin were documented. The other possibility is the "inappropriate" removal rate of the detergent molecules from the detergent–protein complexes. Under such a condition, the stripped and unfolded protein molecules will form intermolecular hydrophobic interactions, which will finally lead to aggregation and/or misfolding. This view could be evaluated through analyzing the microenvironment of the refolded and the partially refolded intermediates by using the ANS fluorescence probe [32]. As shown in Fig. 4, variable concentrations (5, 10, and 15 mg/ml) of the molecular tube did not have measurable effects on the fluorescence intensity profiles of the probe when it is mixed with native ALP and/or CA samples. However, the fluorescence intensity of the probe in the mixture of denatured ALP and/or CA samples and different amounts of the molecular tube (5, 10, and 15 mg/ml) significantly varied. Based on Fig. 4, the fluorescence intensity profiles of the samples in the presence of 5 mg (for ALP) and 15 mg (for CA) of molecular tube are closer to native ALP and CA fluorescence profiles, meaning that at optimal molecular tube concentrations the refolded ALP and CA

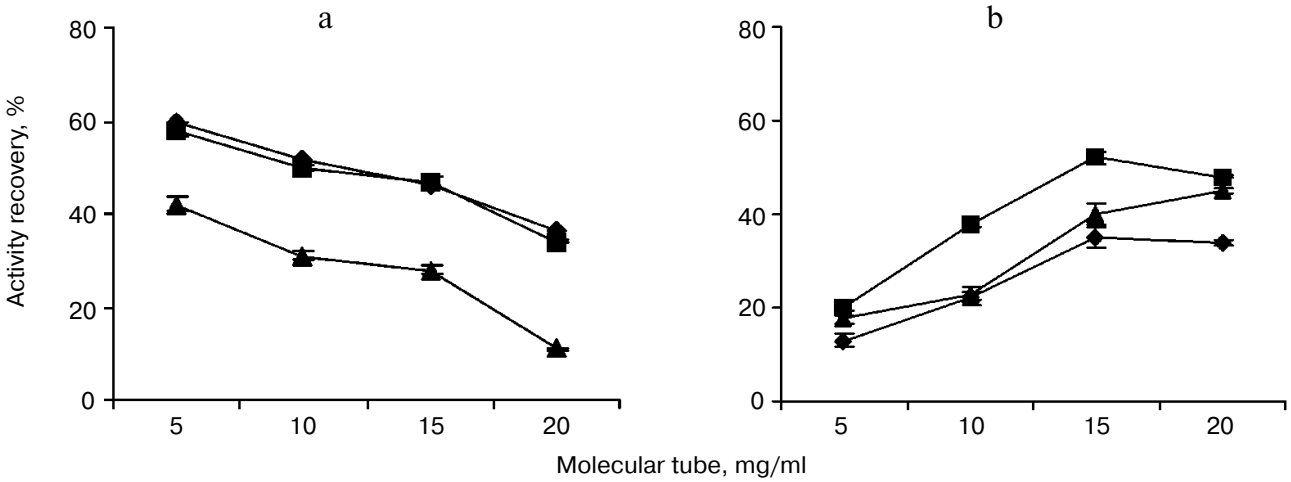


**Fig. 3.** Relative changes in the recovered enzymatic activities of refolded ALP (a) and CA (b) as a function of variable concentrations of CTAB and the molecular tube. CTAB concentrations (mM): 1 (rhombs); 2 (squares); 4 (triangles). Molecular tube concentrations: 5-20 mg/ml.

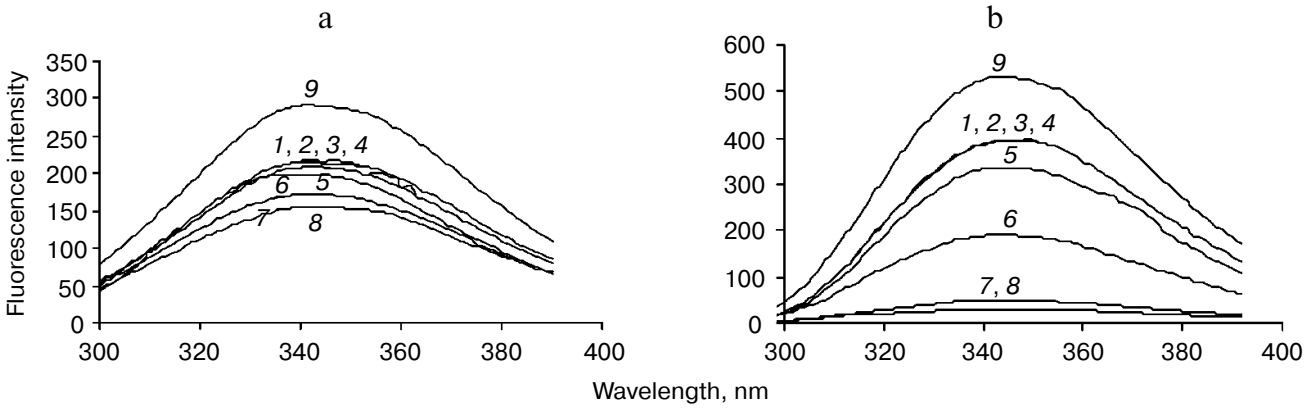


**Fig. 4.** Intrinsic fluorescence spectra of the refolded ALP and CA in the presence of α-CD and different amounts of the molecular tube (a and b). For experimental details, see "Materials and Methods". Curves: *1*a, *1*b) native ALP and CA samples, respectively; *2*, *3*, *4*) native ALP and/or CA fluorescence profiles in the presence of 5, 10, and 15 mg/ml of the molecular tube, respectively; *6*, *7*, *8*) refolded ALP and/or CA fluorescence profiles in the presence of 5, 10, and 15 mg/ml of the molecular tube, respectively; *9*) ALP–detergent and/or CA–detergent complexes.

samples have gained almost similar microenvironments as their respective native forms. However, the fluorescence profiles of ALP in the presence of 10 (Fig. 4a, curve *7*) or 15 mg (Fig. 4a, curve *8*) of the molecular tube significantly varied with respect to the fluorescence profiles of the native (curve *1*) or the refolded sample (curve *6*). In the case of CA, non-optimal levels of the molecular tube created more microenvironmental deviations (Fig. 4b). These fluorescence profiles clearly indicate that detergent stripping from the detergent–protein complexes with non-optimal concentrations of the molecular tube might affect the stripping rates, which in turn will lead to build up of unfolded hydrophobic zones quite suited for intermolecular interactions. These intermolecular hydrophobic interactions are known to be responsible for misfolding and aggregation in the process of refolding [33].

**Influence of protein concentration on the reactivation yield.** As reported previously [13, 15], the net refolding yield in the liquid phase artificial chaperone assisted refolding system was dependent on the protein concentration: the higher protein concentration the lower refolding yield. Our data presented in Fig. 5 clearly indicated that a similar pattern exists in solid-phase artificial chaperone assisted technique too. The maximum activity recoveries for ALP (61%) and CA (51%) were obtained at 0.12 mg/ml of ALP and 0.03 mg/ml of CA, when using 5

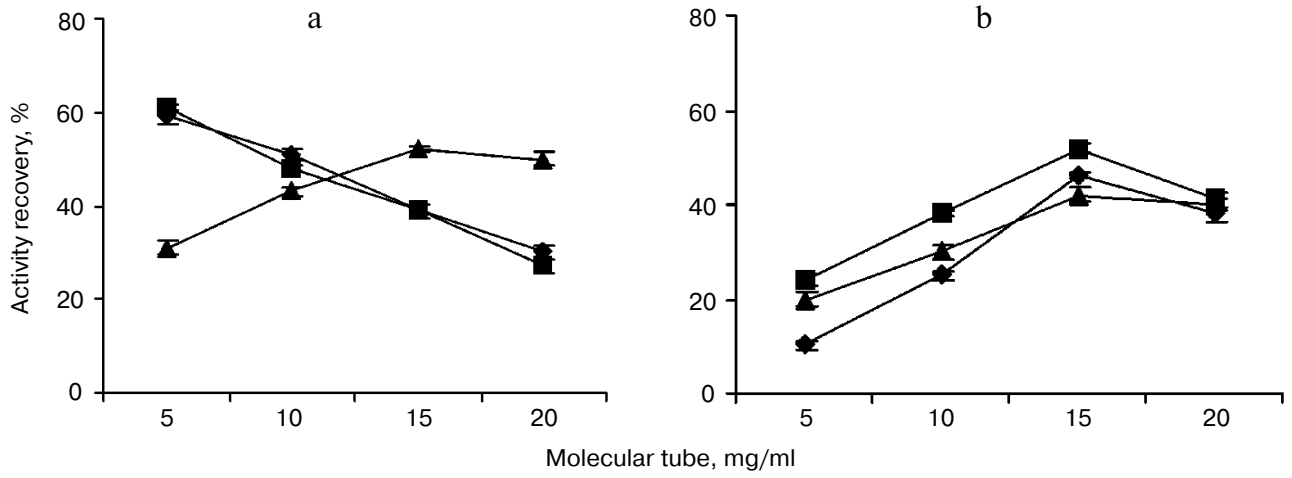


**Fig. 5.** Effect of different concentrations of ALP (a) and/or CA (b) on the refolding yields in the presence of variable amounts of the molecular tube (0-20 mg/ml). ALP concentrations (mg/ml): 0.06 (rhombs); 0.12 (squares); 0.2 (triangles). CA concentrations (mg/ml): 0.015 (rhombs); 0.03 (squares); 0.06 (triangles).

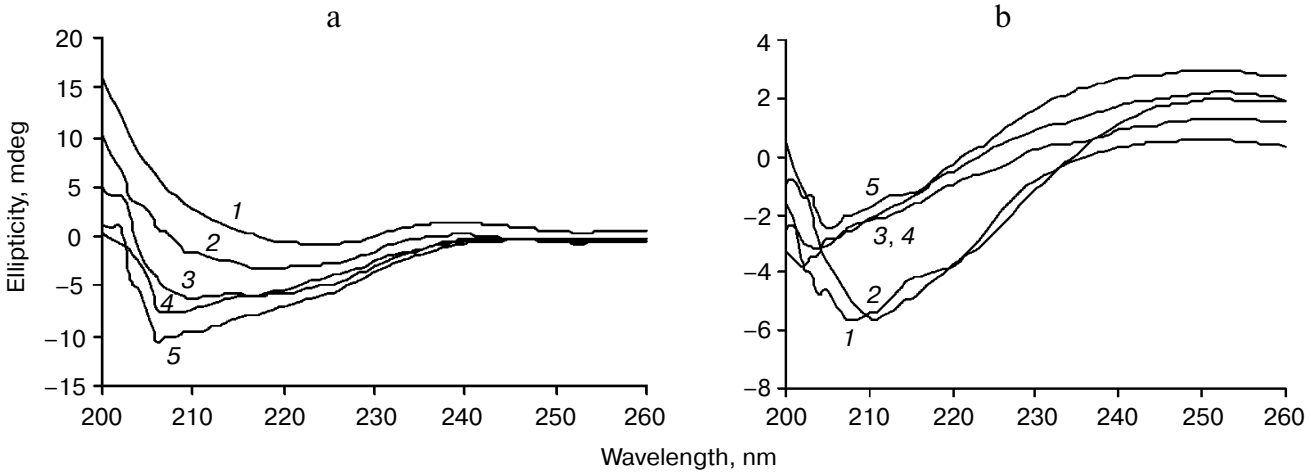


**Fig. 6.** Far-UV circular dichroism spectra of refolded ALP (a) and CA (b) in the presence of different amounts of the molecular tube. Curves in (a) and/or (b): *1*) native enzyme; *2*) refolded enzyme in the presence of α-CD; *3*, *4*, *5*) refolded enzyme in the presence of different amounts of the molecular tube (5, 10, and 15 mg/ml, respectively).

and 15 mg/ml of molecular tube, respectively. At higher protein concentrations, the renaturation yields of ALP and CA dropped to 30 and 40%, respectively. As indicated in Fig. 5, with increasing protein concentration, the required molecular tube to achieve maximum renaturation also increased, while the overall refolding yield decreased, probably due to interferences created for mass transfers at higher resin concentrations.

**Circular dichroism analysis.** To establish a correlation between the structural level and the refolding efficiency in the presence of new stripping agent, we evaluated the secondary structural variation of the chemically denatured ALP and CA under various refolding conditions. Figure 6 shows the far-UV circular dichroism spectra in 200-260 nm range for native ALP and CA (curve *1*), refolded samples in the presence of α-CD (curve *2*), and for the solid-phase artificial chaperone refolded samples (curves *3-5*). According to these graphs, the secondary structural features of the refolded samples in the presence of 5 and 15 mg molecular tube are very close to that of the native ALP and CA, respectively. However, at lower molecular tube contents (e.g. 5 mg), for CA, the far-UV circular dichroism spectrum of the refolded sample has less negative ellipticity attributed to lower α-helix content and for refolded ALP in the presence of 15 mg molecular tube, the far-UV circular dichroism spectrum of the refolded

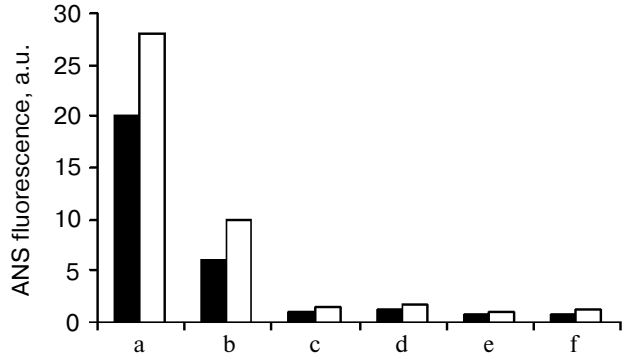


**Fig. 7.** Fluorescence intensity variation of denatured ALP (dark columns) and CA (light columns) while undergoing the refolding process in the presence of ANS. The ANS solution (100 μM) was added to the refolding environment of each sample. Samples: a) detergent–protein complexes; b) refolded ALP and CA at 5 and 15 mg molecular tube, respectively; c) refolded ALP and CA in the presence of α-CD; d) denatured proteins; e) native enzymes; f) free ANS.

sample has more negative ellipticity attributed to higher α-helix content [17]. Based on these observations, it appears that optimal molecular tube value probably minimizes the life time of the partially stripped protein–detergent complexes and consequently the intermolecular interactions will be weakened or not formed. Suboptimal molecular tube values, on the other hand, will allow the refolding intermediate complexes to live long enough to associate with each other leading to decreased renaturation yields.

**Intrinsic fluorescence analyses.** As shown in Fig. 4, the samples refolded in the presence of optimal value of the molecular tube (5 mg, curve *6* in Fig. 4a; and 15 mg, curve *6* in Fig. 4b) possess tertiary structures very close to those of the native ALP and CA samples, respectively. However, suboptimal quantities of the molecular tube (lower or higher than 5 and 15 mg for ALP and CA, respectively) cause significant variation in the intrinsic fluorescence profile of the refolded samples, indicating that the tertiary structures are different from those of the native samples. These conformational variations have been attributed to too slow or too fast detergent stripping which will end up in misfolding of the samples [22, 34]. The intrinsic fluorescence data presented in this report indicate that the tertiary structures in the protein–detergent complexes are also different from the native tertiary structures. We also attempted to determine the structural characteristics of the refolding intermediates of ALP and CA using the ANS fluorescence probe. Figure 7 shows the

**Table 2.** Renaturation of GuHCl-denatured ALP and CA in the presence of different kinds of additives in two refolding approaches

| Additives | | Artificial chaperone assisted mode | | | Dilution additive mode | |
|---|---|---|---|---|---|---|
| | | Activity recovery, % | Inhibition of aggregation, % | Residual of CTAB, % | Activity recovery, % | Inhibition of aggregation, % |
| ALP | Buffer | 43 ± 1.5* | | | 40 ± 2.2 | |
| | α-CD (16 mM) | 75 ± 2.7** | 70 ± 1.2 | | 38 ± 1.2 | 52 ± 2.2 |
| | CTAB (1 mM) | 40 ± 1.6 | 43 ± 2.4 | | 40 ± 1.5 | 42 ± 2.4 |
| | Polymer (5 mg/ml) | 61 ± 1.2*** | 80 ± 0.7 | 23 ± 1.2 | 15 ± 1.6 | 60 ± 1.5 |
| | Polymer (10 mg/ml) | 51 ± 2.4 | 84 ± 1.4 | 15 ± 1.5 | 20 ± 2.3 | 65 ± 1.7 |
| | Polymer (15 mg/ml) | 46 ± 1.4 | 91 ± 1.6 | 14 ± 1.9 | 22 ± 2.2 | 60 ± 1.8 |
| | Polymer (20 mg/ml) | 36 ± 1.9 | 91 ± 2.2 | 9 ± 2.2 | 18 ± 1.8 | 72 ± 1.4 |
| CA | Buffer | 35 ± 1.2* | | | 32 ± 2.3 | |
| | α-CD (16 mM) | 75 ± 1.9** | 91 ± 1.2 | | 62 ± 2.0 | 70 ± 0.7 |
| | CTAB (1 mM) | 42 ± 1.6 | 58 ± 2.2 | | 53 ± 1.5 | 58 ± 2.1 |
| | Polymer (5 mg/ml) | 20 ± 1.3 | 70 ± 0.7 | 38 ± 2.2 | 8 ± 1.8 | 70 ± 1.2 |
| | Polymer (10 mg/ml) | 38 ± 2.2 | 78 ± 1.4 | 20 ± 1.7 | 12 ± 1.2 | 78 ± 2.1 |
| | Polymer (15 mg/ml) | 52 ± 1.5*** | 93 ± 1.8 | 23 ± 1.9 | 15 ± 1.6 | 83 ± 1.9 |
| | Polymer (20 mg/ml) | 48 ± 1.7 | 100 ± 1.9 | 12 ± 1.4 | 11 ± 1.8 | 81 ± 1.6 |

Note: Each value represents the average of three independent measurements ± SD. Differences between values * vs. ** and vs. *** were statistically significant ($p < 0.01$).

fluorescence profile of the mixture of ANS and the refolding intermediates during the refolding of ALP and CA from the denatured state. The $\lambda_{max}$ of ANS shifts to 490 and 440 nm in the presence of the refolding ALP and CA, respectively, along with an increase in the fluorescence intensities. These results clearly indicate that upon denaturation, hydrophobic surfaces of ALP and CA become more exposed to ANS and therefore lead to enhanced fluorescence intensities. Upon gradual refolding, the fluorescence intensity will return back to that of the native sample. According to Fig. 7, the fluorescence intensity of the protein–detergent complexes is much higher than the fluorescence intensity of native ALP and CA samples, indicating less fluorescence quenching of the tryptophan side chains in the protein–detergent complex than in the native enzyme forms.

**Assisted ALP and CA renaturation in dilution additive mode using the molecular tube.** Beside artificial chaperone assisted refolding techniques, denatured proteins can be assisted to refold in dilution additive mode using various refolding aids such as some amino acid [3-6], sugars [35], polyethylene glycol [7-9], and cyclodextrins [12, 14]. The denatured ALP and CA samples were diluted directly with the refolding buffer containing 5-20 mg/ml molecular tube. As shown in Table 2, no refolding assistance was observed compared to the respective control samples (without the molecular tube), indicating that this α-CD polymer was unable to assist ALP and CA renaturation through the dilution additive approach. As we have previously suggested [36], the cyclodextrin molecules, especially at high concentrations, create hydrophobic areas within the refolding media. Such environments probably shield the hydrophobic surfaces on denatured protein molecules, therefore depressing the intermolecular forces involved in aggregate formation. This situation will encourage the intramolecular hydrophobic interactions responsible for proper refolding of the intermediates. Polymerization of cyclodextrin molecules into an insoluble molecular tube apparently prevents the creation of such an environment and therefore the "folding aid" effect of cyclodextrin is destroyed upon polymerization.

In conclusion, the results of this investigation indicate that carbonic anhydrase as a monomeric protein and alkaline phosphatase as a dimeric protein can be refolded with moderately high efficiency using the solid-phase artificial chaperone assisted refolding approach. Regardless of lower refolding yields, solid-phase artificial chaperone assisted technique is more suited for industrial applications due to easier and faster separation of the stripping agent from the refolded samples and the recycling of the stripping agents. These advantages will certainly result in lower production costs. However, further work is required to enhance the refolding yields either through defining more efficient refolding environments or by synthesizing other derivatives of α-CD with more efficient stripping capabilities.

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