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# Affinity Purification and Characterization of *E. coli* Molecular Chaperones

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# AFFINITY PURIFICATION AND CHARACTERIZATION OF *E. COLI* MOLECULAR CHAPERONES

by

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# ABSTRACT

Affinity Purification and Characterization of *E. coli* Molecular Chaperones

by

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The molecular chaperones are a group of proteins that are effective *in vitro* and *in vivo* folding aids and show a well documented affinity for proteins lacking tertiary structure.

Heat-induced *Escherichia coli* BL21 cell lysate (10 mg protein) was applied to immobilized α-casein (45 mg/g beads) or β-casein (30 mg/g beads) column. After removing a majority of nonspecifically bound proteins with 1 M NaCl, the molecular chaperones were eluted with cold water, 1 mM Mg-ATP, or 6 M urea. Western analysis identified five *Escherichia coli* molecular chaperones including DnaK, DnaJ, GrpE, GroEL, and GroES. Among samples, ATP eluates showed the highest chaperone purity of 80-87% followed by cold water eluates with 62-68% purity. The β-Casein column showed a higher binding capacity than the α-casein column since β-casein urea eluates

contained 3.18 mg total protein (or 58% chaperone) compared to α-casein urea eluates with 2.68 mg total protein (or 32% chaperone). For strain comparison, *Escherichia coli* NM522 eluates showed more unidentified proteins in cold water eluates from both affinity columns.

Chaperones were induced from BL21 strain with three treatments: heat shock at 39 °C, heat shock at 42 °C, and alcohol shock with 3% ethanol (v/v). Lysates were applied to an immobilized β-casein (30 mg/g beads) column. The molecular chaperones were eluted with cold water or 1 mM Mg-ATP after washing with 1 M NaCl. The purity of eluted chaperones was 58% with cold water and 100% with Mg-ATP. The treatment at 42 °C was the most efficient for chaperone induction with highest chaperone yield of 1.0 mg among samples. Refolding denatured carbonic anhydrase B enzyme in the presence of Mg-ATP resulted in a 97% recovery of heat-denatured enzyme and a 68% recovery of chemically denatured enzyme.

It was concluded that the novel casein affinity chromatography is a rapid and efficient method for purification of chaperone. The affinity purified chaperones were effective *in vitro* folding aids.

(157 pages)

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**Abbreviation Key**

| | |
|---|---|
| APCP | affinity purified chaperones |
| ACP | artificial chaperones |
| CAB | carbonic anhydrase B |
| CAB (N) | native CAB |
| CAB (H) | heat denatured CAB |
| CAB (C) | chemical denatured CAB |
| CLP | caseino lytic protease or chaperone linked protease |
| CN | casein |
| CPG | controlled-pore glass |
| CTAB | cetyltrimethylammoniumbromide |
| DMSO | dimethylsulfoxide |
| $LON^-$ | genetically deficient in lon protease gene |
| PMSF | phenylmethylsulfonylfluoride |
| PNPAc | p-nitrophenyl acetate |

# CHAPTER I

# INTRODUCTION

The art of protein folding or refolding is essential to the biotechnology industry with respect to *in vitro* protein synthesis of recombinant protein in a heterologous host (47). Recombinant proteins are currently used as therapeutic aids and enzymes in food processing. Soluble general and specific folding aids have enhanced the folding of proteins from a denatured state or during protein synthesis by suppressing protein aggregation (14, 90, 91). Molecular chaperones are found in both eukaryotes and prokaryotes and are involved in protein folding, translocation, translation initiation, gene expression, and growth control. They prevent aggregation, assist refolding, and mediate degradation of misfolded proteins.

The optimum folding aid would be cost effective, reusable without reduction in efficiency, inhibit protein aggregation without adversely affecting formation of native protein, easily separated from the native protein after completion of folding, and general enough to work for many proteins (14). An ideal situation would be to immobilize molecular chaperones to enhance protein folding but this may not be possible because of the cascade or concerted action exhibited by chaperones and their oligomeric composition.

Current methods of purifying chaperones involve overexpression of the

chaperones from either heat-stress cells or from a multicopy plasmid in *E. coli*. Several affinity purification methods have been described to purify molecular chaperones from stressed cells (39, 100). These affinity purification methods showed low recovery and specificity with respect to the *E. coli* molecular chaperones. Denatured proteins or chaperones immobilized onto a solid matrix were capable of purifying a small fraction of the molecular chaperones and no one method was successful in purifying more than 3 different chaperones.

It has been suggested that chaperones might recognize unfolded polypeptide chains, the molten globule conformation, secondary structures or hydrophobic sequences (88). Unlike most globular proteins, the milk caseins are not found individually in milk, but form large quaternary complexes known as casein micelles. The caseins are amphiphilic phosphoproteins with characteristics similar to the molten globule folding intermediate. This research studied the development of affinity chromatography techniques, using caseins ($\alpha$- and $\beta$-caseins) that are recognized by molecular chaperones due to caseins characteristics which are similar to the molten globule folding intermediate.

Although molecular chaperones have been purified and researched extensively in prokaryotes and eukaryotes, current purification procedures do not yield sufficient quantities of active molecular chaperones to investigate the refolding of denature

proteins. This study was effective in the purification of five molecular chaperones in suitable amounts to investigate protein refolding. The casein affinity techniques for purifying chaperones could improve production of recombinant proteins and enzymes for therapeutic and processing uses.

Chapter II outlines the characterization of interactions and dissociation between molecular chaperones and immobilized α- and β-casein matrices from two *E. coli* strains. Chapter III describes the affinity purification of *E. coli* molecular chaperones induced with various treatments from BL21 using immobilized β-casein matrix. Purified molecular chaperones were characterized with respect to refolding of CAB (denatured carbonic anhydrase B). In the Appendices, affinity purification of molecular chaperones from salt induced *E. coli* and an attempt at the purification of Clp (caseino lytic or chaperone linked protease) from *E. coli* are described.

The folding of proteins from their initial unstructured state to their mature form has long been known to be promoted by other proteins known as "molecular chaperones." The molecular chaperones play roles in preventing aggregation, assisting refolding and mediating degradation of misfolded proteins (2, 44). The molecular chaperone family in *E. coli* includes heat shock 70 (Hsp 70), heat shock 60 (Hsp 60), heat shock 90 (Hsp 90), and heat shock100 (Hsp 100). In addition to natural chaperones, artificial chaperones have been developed to facilitate protein refolding by mimicking the basic functional

features of chaperones (91). Current methods of purifying chaperones involve overexpression of the chaperones from heat-stress cells or from a multicopy plasmid in *E. coli*. Chaperones recognize unfolded polypeptide chains, the molten globule conformation, or hydrophobic sequences. The caseins are amphiphilic phosphoproteins with characteristics similar to the molten globule folding intermediate. Because of their unique characteristics, caseins have been incorporated into chaperone mediated refolding studies (71). The art of protein folding or refolding is essential to the biotechnology industry with respect to *in vitro* protein synthesis of recombinant proteins to successfully produce proteins and enzymes for therapeutic and processing use (47).

## *E. COLI* MOECUALAR CHAPERONE FAMILY

### *Major Chaperone Families: Hsp 70, Hsp 60, and Hsp 90*

The existence of most molecular chaperones under normal growth conditions is essential for cell viability (85). Most chaperones are expressed constitutively in cells, with increased expression with response to stress such as high or low temperature, ethanol shock, osmotic shock, and pH shock (118). In *E. coli*, members of the Hsp 70 family include DnaJ, DnaK, and GrpE, the Hsp 60 family includes GroES and GroEL, and the Hsp 90 family includes HtpG (37, 45). Eukaryotes and other prokaryotes contain

proteins that are homologous and have similar functions as the *E. coli* chaperones. Hsp 70 proteins are involved in stabilizing newly synthesized proteins and protein translocation, folding and assembly (26, 61). The Hsp70 proteins bind newly synthesized proteins *in vivo* and proteins exhibiting little secondary and tertiary structure *in vitro* (28, 85). *E. coli* Hsp 70, DnaK, binds ATP and possesses ATPase activity that is regulated by DnaJ and GrpE. In contrast, GroEL and GroES promote protein folding and assembly by recognizing a protein folding intermediate such as the molten globule (69, 92). Hsp 90 is reported to associate with kinases, steroid receptors, and sigma factors (132). In these different interactions, Hsp 90 seems to stabilize target proteins in an inactive, partially unfolded or unassembled state and can be termed a general chaperone (77).

DnaK, GrpE, and HtpG are monomeric proteins with molecular weights of 70 kDa, 20 kDa, and 71 kDa, respectively. DnaJ is a homodimer of 41 kDa per monomer. GroEL has a molecular weight of approximately 802 kDa and is composed of 14 identical subunits of 57 kDa each (28, 37). Electron micrographs of GroEL show a hollow cylinder with a cavity that spans the sevenfold axis of symmetry. There are several models explaining the interaction of the protein substrate with GroEL, which include both encapsulation and surface contact of the protein (118). GroES is a heptameric ring of identical 10 kDa subunits and can bind on either end of the GroEL cylinder (69).

Chaperones may mediate the correct folding and assembly of polypeptides by working in a sequential mechanism on newly synthesized peptides as described in Fig. 1 (85). A model first described by Langer et al. (62) and Martin et al. (70) then modified by Pfanner (85) shows that DnaK, in cooperation with DnaJ, binds to exposed hydrophobic segments of the nascent polypeptide chain or proteins lacking tertiary structure is transferred to the GroEL/ES system where the protein can fold, again with the expense of ATP. A single round or multiple rounds of interaction with these chaperones is required to complete the folding process depending on the protein substrate.

Since molecular chaperons do not change protein folding pathways but increase recovery yields by preventing aggregation side reactions, it has proposed that they should be present in the refolding buffer in at least equimolar concentrations before addition of denatured substrate (118). In *E. coli*, two molecular chaperone systems, DnaK-DnaJ-GrpE and GroEL-GroES have been studied extensively. DanK alone or the complete DnaK-DnaJ-GrpE system is able to improve refolding yields in the presence of ATP if Therefore, when a substrate is larger than 65 kDa, DnaK, a monomer, could form a 1:1 molar complex with GrpE, a monomer and a 1:2 molar complex with DanJ, a dimer. For a substrate smaller than 50-60 kDa, GroES, a heptamer, could bind to GroEL, a tetradecamer by formation of a 1:2 molar complex.

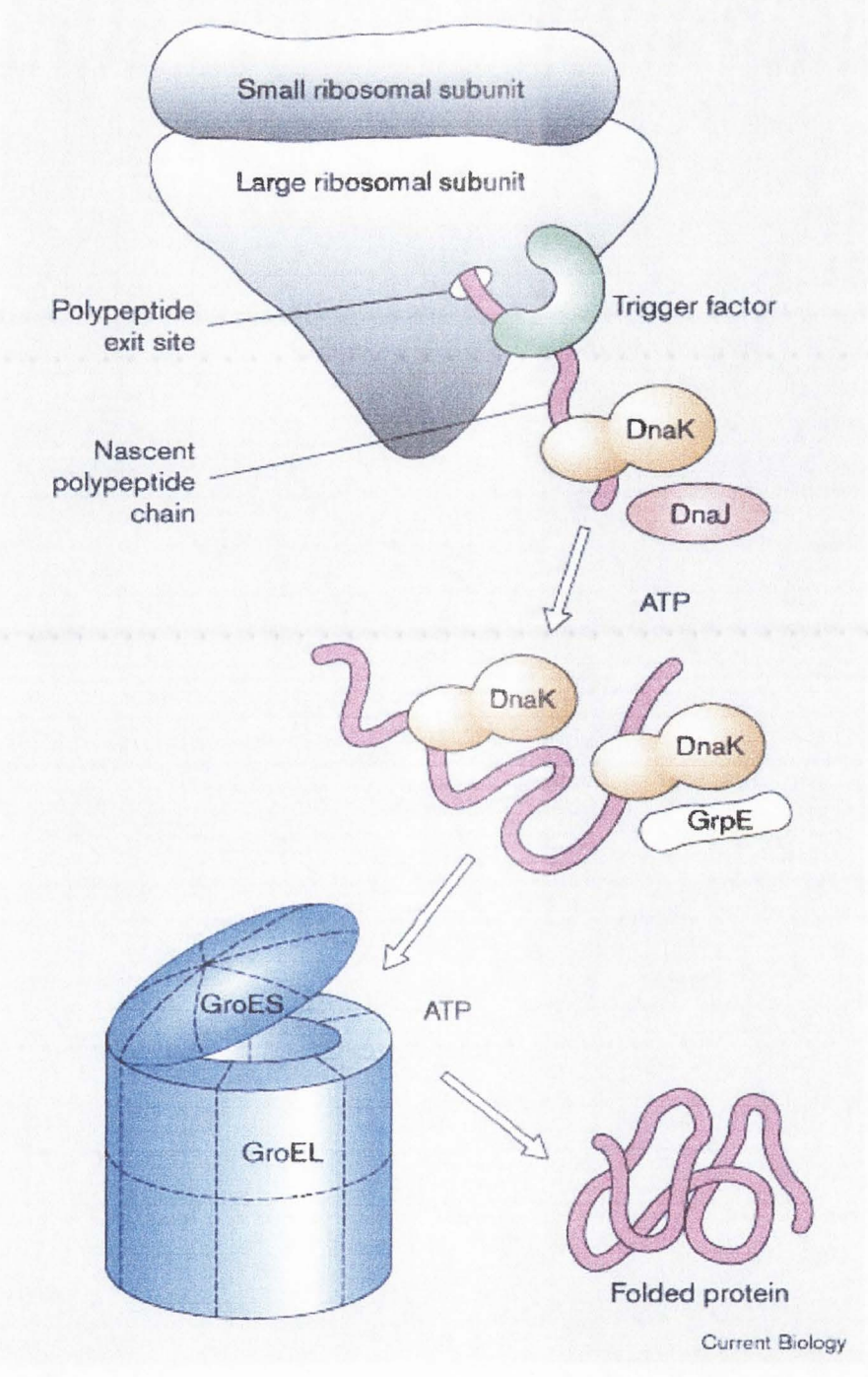


**FiG. 1.** Folding pathway for a model protein involving Hsp 70 and Hsp 60 families in the *E. coli* cytosol (85).

The division of labor between GroEL/GroES and the DnaJ/DnaK/GrpE complexes is not completely known, but several authors have hypothesized the sequential interaction of a newly synthesized proteins with molecular chaperones (70, 71). The chaperones cooperate in protein folding while expressing differential specificity for structural features of a polypeptide chain. It has been suggested that chaperones might recognize unfolded polypeptide chains, molten globule conformation, secondary structure or hydrophobic sequences according to Richerme and Kohiyama (88) and Gottesman and Hendrickson (37). The preferential substrates for DnaK are peptides or unfolded proteins containing internal hydrophobic residues and terminal polar residues. GroEL has been found to bind to the molten globule form of many proteins (71) and to interact with positively charged side chains (51). The binding of substrate protein to GroEL is probably not dependent on any single feature but is a combination of overall hydrophobicity, net positive charge and secondary structure (37).

The addition of GroEL to denatured phage 22 tailspike protein resulted in release of active protein by the addition of Mg-ATP. The GroEL-protein complex also dissociated without addition of Mg-ATP when cooled to 25°C (9). The dissociation constants (Kds) for unfolded lactate dehydrogenase bound to the various chaperonin complexes were measured. The tightest complex is with GroEL and the weakest with GroEL-MgATP-GroES. All were in the nanomolar range (108, 109). GroEL alone is

sufficient to refold some proteins, other proteins need a combination of GroEL and nucleotide (ATP, CTP, or UTP), while others need GroEL and GroES and nucleotides (37). Other investigators have noted that potassium is required in chaperone mediated refolding (121).

Chaperones prevent the aggregation of partially folded and newly synthesized polypeptides and ensure their proper folding (44). Purified chaperones were applied in refolding studies with denatured enzymes to prove their function (63). *In vitro*, GroEL, GroES (Hsp 60 families), and Mg-ATP promoted the correct folding of rhodanese (80% recovery) (74), rubisco (80% recovery) (41), citrate synthase (28% recovery) (5), firefly luciferases (20% recovery) (8), and glucose 6-phosphate dehydrohenase (90% recovery) (42). DnaJ, DnaK, GrpE (Hsp 70 families), and MgATP were shown to be essential to the reactivation of heat-denatured luciferase (56, 96, 114) and preS2-β-galactosidase (117). GroEL, GroES, DnaK, DnaJ, and GrpE were all necessary for the *in vitro* synthesis of active rhodanese (57) and β-lactamase (117). HtpG (Hsp 90 family) increased citreate syntase refolding from 15% to 40% in an ATP-dependent process (37, 128).

The *E. coli* chaperones have been cloned and used *in vivo* to increase the amount of properly folded recombinant proteins. Overexpression of GroEL and GroES increased the amount of correctly assembled recombinant rubisco (25, 33) and bacterial luciferases

(20). The over expression of both the Hsp60 and Hsp70 families improved the solubility of recombinant kinases (11). The level of soluble human procollagenase in *E. coli* increased 10 fold with the overexpression of GroEL, GroES, and DnaK (64) and reactivation of heat denatured luciferase was abolished in *E. coli* mutants lacking DnaK and DnaJ (96).

*HSP 100 Family (Clp Proteins)*

The Hsp 100/Clp proteins are found in both eukaryotes and prokaryotes (76). The Hsp 100/Clp proteins are strongly induced in response to a variety of stressful conditions and their functions seem specific to conditions of stress (84, 94). The Hsp 100/Clp proteins have roles in many cellular processes including protein reactivation, protein degradation, DNA replication, regulation of gene expression, thermotolerance, inheritance of prion-like factors, and protein translocation through membranes (93, 95). The Clp proteins are important ATP-dependent and chaperone-linked proteases in *E. coli* and named for their capacity to promote the proteolysis of casein (Caseino-Lytic Protease) in vitro (49). The structures of ATP-dependent proteases in *E. coli* are shown in Fig. 2 (106).

There are two kinds of ATP-dependent proteases in *E. coli* including single-chain protease, Lon (La) and two-chain proteases such as ClpAP (Ti), ClpXP, and

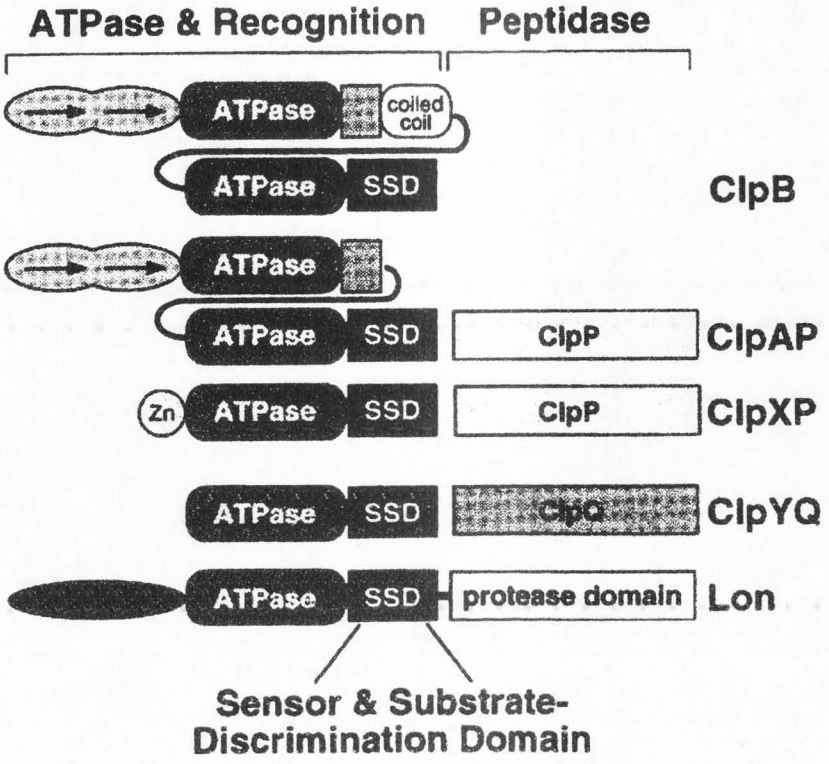


**FiG. 2.** The protein sequences of Lon and the Clp proteins (106).

ClpYQ (HslUV) (4, 36). For the latter proteases, ClpP or ClpQ is the peptidase subunit, and ClpA, ClpX, or ClpYis the ATPase and substrate-binding subunit. The lon and Clp proteases of regulatory ATPase subunits form six-member rings that can bind at both ends of the protease stack (83, 129). The resulting assembly sequesters the protease active sites in a central cavity, preventing inadvertent cleavage of the wrong proteins. This architecture requires that the Clp ATPase subunits act as gatekeepers, recognizing the proper substrates and mediating their delivery to the proteolytic cavern (48). By themselves, ClpA and ClpX can function as disassembly chaperones to catalyze the dissociation of certain multimeric proteins (105, 124). Another Clp ATPase, ClpB, is not known to interact with protease subunits and may function solely as chaperone (130). ClpP is synthesized as a protein of 207 amino acids (106), of which 14 amino acids at the N terminus are autocatalytically removed to yield the mature enzyme (119). The active protein consists of a tetradecamer with molecular weight of 21 kDa and composed of two stacked heptameric rings (106, 119). The structure of ClpP is analogous to the 20 S proteasomes of eukaryotes and Archaebacteria, with multiple active sites residing in the interior of the multimeric rings (53). Presumably, the molecular chaperones unfold the substrate and feed it into the proteolytic chamber of the ClpP tetradecamer, leading to the apparently processive degradation of the substrate (41).

The *E. coli* ClpA is a dimer of 84 kDa subunits and assembles to a hexamer in the presence of ATP. ClpA protein substitutes in vitro for the DnaK/DnaJ/GrpE chaperone machine in activation of the phage P1 RepA replication protein for binding to its recognition sites in the P1 replication origin (48). ClpA also prevents irreversible heat inactivation of luciferase *in vitro* (105). In an ATP-dependent reaction, ClpA translocates substrates from their binding sites on ClpA to ClpP and that substrates can be degraded to the final small polypeptide products following a single round of substrate-binding to ClpAP (67, 73, 98, 105).

*E. coli* ClpX (46 kDa) is known to be involved in two distinct stages of the Mu life cycle (bacteriophage MuA transposase and Mu repressor). As part of the ClpXP protease, it can promote entry of a lysogen into lytic development by degrading the Mu immunity repressor (32, 127), which serves to shut down Mu transposition functions for the establishment and maintenance of lysogeny. ClpX also promotes initiation of Mu DNA replication, a process that does not require ClpP (75). It activates MuA's function of promoting transition to DNA synthesis after MuA's role in recombination has been completed (55). ClpX, *in vitro*, also prevents and reverses heat-induced aggregation of bacteriophage O protein and activates DNA binding by the TrfA replication initiator of plasmid RK2 by converting inactive dimers to active monomers (55, 75).

Electron microscopic studies have shown that the ClpA ring binds to the 7-fold symmetric ClpP component, which itself is arranged in a barrel-like double-ring structure. Similar structures are seen with ClpXP and ClpYQ complexes (35, 125). Thus, Clp ATPase components are situated in an ideal position to regulate the entry of specific substrates into the aqueous core of ClpP. ClpA and ClpX interact directly with proteins and function in substrate discrimination (103). ClpX degrades such proteins as *E. coli* RpoS (97), phage P1 PhD (65), and λO protein (35, 125), whereas ClpA specifically degrades *E. coli* MazE (1) and engineered N-end rule substrates, such as Leu-galactosidase (120).

ClpB protein is essential for survival of *E. coli* at high temperatures (107). ClpB functions as a chaperone in reactivation of aggregated proteins. ClpB has a tetrameric ring structure of the 93 kDa subunit with a central cavity. ClpB has ATPase activity that is stimulated 5-10 fold by casein (95). It is also activated by insulin, but not by other proteins, including globulin and denatured bovine serum albumin (130). In the absence of salt and ATP, the ClpB proteins show a high tendency to form a heptamer but dissociate into oligomers with smaller sizes, depending on salt concentration (above 0.2 M NaCl) (36, 76).

In addition to ClpP in *E. coli*, Lon is also an ATP-dependent protease and composed of a tetramer with identical 87 kDa subunits. Lon plays an important role in

cellular processes by modulating the availability of certain regulatory proteins or degrading abnormally folded proteins (36). Regulatory proteins, such as the cell division inhibitor SulA or RcsA, involved in capsule synthesis, were shown to be degraded by the Lon protease (106).

*Artificial Chaperones*

Several techniques that facilitate protein refolding by mimicking the basic functional features of chaperones have been developed. Rozema and Gellman (17, 90, 91) have used detergents and cyclodextrins to duplicate the two-step mechanism of action of the GroEL /GroES chaperones system in the refolding of carbonic anhydrase B, citrate synthase, and lysozyme. This has been described as the "artificial chaperone" method, in which a pair of small molecules assist protein folding in vitro (Fig. 3) (43). In the first step of artificial chaperone protocol (capture), GdmCl-denatured protein is diluted to a non-denaturing GdmCl concentration in the presence of a detergent (e.g., CTAB, STS, or Trition X-100), resulting in the formation of a stable protein-detergent complex. The bound detergent prevents both aggregation and native folding, presumably by shielding solvent-exposed hydrophobic patches. Since this process also prevents any "legitimate" refolding, it is analogous to the trapping of non-native proteins within the central cavity of the GroEL toroid.

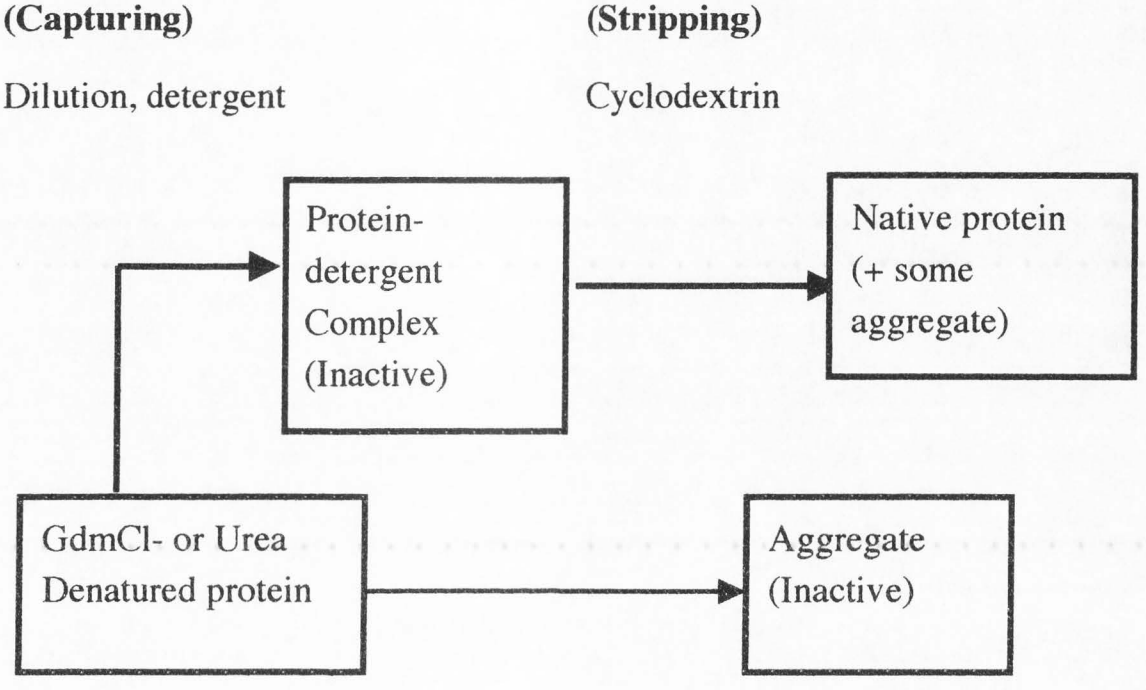


**FiG. 3.** Schematic diagram of artificial-chaperone assisted protein refolding by recognizing exposed hydrophobic patches (43).

In the second step (stripping), a cyclodextrin is added to the protein-detergent complex, causing the removal of the detergent from the protein, and concomitant renaturation. This is superficially similar to the effect of interactions between GroEL-polypeptides complexes and ATP/GroES that stimulate the release of bound proteins to permit their correct isomerization. This method previously has been shown by Couthon *et al.* (15) to facilitate refolding of MM-creatine kinase.

Another approach that mimics the aggregation suppression function of molecular chaperones is the "temperature leap" tactic developed by Xie and Wetlaufer (131) to refold carbonic anhydrase II. In this case, unfolded proteins are diluted in a refolding buffer held at low temperature to suppress interactions between hydrophobic segments in the protein chain and favor the conversion of aggregation-prone folding intermediates into partially folded but non-aggregating species. Shifting the refolding mixture to higher temperatures allowed productive folding to occur. Finally, Stempfer and Neugebauer (110) have documented the successful refolding of A-glucosidase fused to a hexa-arginine extension upon noncovalent immobilization on polyionic matrices. This process is likely to create a microenvironment that favors proper folding since matrix-bound folding intermediates are prevented from interacting with each other to give rise to aggregation reactions.

## PURIFICATION OF MOLECULAR CHAPERONES

### *Major Chaperone Families; Hsp70, Hsp60, and Hsp90*

Current methods of purifying chaperones in *E. coli* involves overproducing the heat shock transcription factor sigma 32, or inducing the heat-shock response by increasing cell growth temperature, adding ethanol, or other agents to the growth medium (80, 118).

The amount of GroEL in *E. coli* cells has been estimated at 1.6% of the total protein or 2.75 mg/ml which increases 10-fold upon heat shock (22). Purification of GroEL and GroES can include either centrifugation in a sucrose gradient after sonication or ammoniun sulfate precipitation followed by ion-exchange (3, 5, 52, 108, 121) or size exclusion chromatography (18, 52, 108, 123). The protein purity was increased by repeated ion-exchange (3, 5) or hydrophobic interaction chromatography (18, 27, 101, 121). Sherman and Goldberg (101) purified 13% of the total cellular GroEL and GroES using CRAG affinity column which contains protein A and unfolded domains.

The Hsp 70 family accounts for about 1% of total cellular protein even under normal growth conditions (89). After ammonium sulfate precipitation or sonication, DnaK, DnaJ or GrpE were purified from *E. coli* cell lysate with a series of chromatography steps including ion exchage (29, 38, 81, 114, 122), size exclusion (16,

23, 56, 82), and hydrophobic interaction chromatography (12, 56, 79). Kubo et al. (56), Szabo et al. (114), and Liberek et al. (66) purified three proteins of the Hsp70 family with the combination of several chromatography steps including gel filtration, ion exchange, reverse phase, and affinity techniques. For the affinity purification, immobilized ATP (56, 78, 81), gelatin (78), Glutathion-S-sepharose (82), or Ni-NTA (38) were utilized to purify DnaK from *E. coli* cell lysate. In addition to DnaK, DnaJ was affinity purified using Cellulose phosphate (56) and Red A agarose (134). Histidine (56) or Dnak (79) was immobilized for the affinity purification of GrpE.

The Hsp 90 or HtpG is one of the most abundant cytosolic proteins in prokaryotes, amounting to 1% of soluble protein even in the absence of stress (7). HtpG was purified using gel filtration, ion exchage, and ATP-agarose affinity chromatography (74, 126).

Several affinity purification methods have been described to purify molecular chaperones from stressed cells. Sherman and Goldberg (102) expressed a chimeric protein in *E. coli* containing truncated cro repressor and β-galactosidase linked to protein A (CRAG). The protein A moiety of the chimeric protein was able to bind immobilized IgG while the terminal region associated with heat-shock proteins. A fraction of the cellular DnaK and GroEL associated with CRAG *in vivo* and could be dissociated from immobilized CRAG by ATP. Immobilized CRAG was then used as an affinity ligand to

purify chaperones from heat stressed cells, but only a small fraction of the cellular DnaK (10-15%), GrpE (3%) and GroEL (7-10%) bound to CRAG *in vitro* (102) and were released with MgATP. This method could not detect DnaJ nor GroES in the affinity pruified sample. Evers et al. (21) used immobilized denatured alcohol oxidase and ATP to purify chaperones from *Hansenula polymorpha* and *Saccharomyces cerevisiae* but did not report recovery rates. Purified GroEL associated with the immobilized protein but quantitative elution only occurred with 8 M urea and did not occur with ATP. Members of the Hsp 70 family or Hsp 90 have been affinity purified with immobilized ATP/ADP (39) or immobilized unfolded polypeptide (100). Several large molecular weight proteins along with the Hsp 70 protein were released with ATP from both affinity matrices.

However, These affinity purification methods showed low recovery and specificity with respect to the *E. coli* molecular chaperones. The denatured proteins, ATP or other chaperone immobilized on solid matrix were capable of purifying a small fraction of the molecular chaperones and no one method was successful in purifying more than 3 different chaperones.

*HSP 100 Family (Clp Proteins)*

Hsp 100 or Clp protein (chaperone-linked protease or casein lytic protease) was overexpressed from a multicopy plasmid in *E. coli* or from severe stressful conditions

such as heat exposure at 46-48 °C, salt (4% w/v), or ethanol (5% v/v) (31). ClpP constitutes about 2% of the cellular protein (73). The amounts of ClpA, ClpX or ClpB in *E. coli* has been estimated at 1% of the soluble cellular proteins (41, 73, 130).

Purification of Clp proteins includes either centrifugation in a sucrose gradient, or sonication (41, 106) and followed by ammoniun sulfate precipitation or salt precipitation (48, 73, 125) followed by ion-exchange chromatography (67, 72, 83) or size exclusion (104, 106, 130). The protein purity was increased by repeated ion-exchange (41, 67, 72) or hydrophobic interaction chromatography (34, 83). Several affinity purification methods have been developed to purify ClpP using Ni-NTA agarose (106) or phosphocellulose column (72), ClpA using phophocellulose column and heparin agarose (67, 99), and ClpB using heparin agarose (54, 130). For the purification efficiency of Clp proteins, ClpP or ClpA recovery was 44-47% (54, 73, 83) and ClpB recovery was 69% (54, 130).

## MILK PROTEINS AND MOLECULAR CHAPERONES

### *Characteristics of Milk Proteins*

Milk contains approximately 36 g protein per liter of fluid milk of which 28 g is casein and the remainder is mostly whey proteins. The casein fraction is composed of

$\alpha_{S_1}$-, $\alpha_{S_2}$-, β- and κ-casein while the two major whey proteins are α-lactalbumin and β-lactoglobulin. $\alpha_{S_1}$-Casein is present at 12-15 g per liter, $\alpha_{S_2}$-casein at 3- 4 g/liter, β-casein at 9-11 g/liter and κ-casein at 3-4 g/liter (111). The primary structures of all the major milk proteins are known, but the three dimensional structures of only the whey protein have never been determined because the caseins cannot be crystallized (112).

Unlike most globular proteins, caseins are not found individually in milk but form a large protein complex known as the casein micelle. The caseins are amphiphilic phosphoproteins with distinct hydrophobic and hydrophilic regions. Secondary structure predictions by sequence analysis, CD spectra and Raman spectra indicate the presence of α-helix, β-structure and β-turns (112).

Because of their amphiphilic structure and the large size of their hydrophobic domains, individual casein monomers cannot sufficiently remove their hydrophobic surfaces from contact with water. Therefore, they tend to associate into polymers, in the case of isolated individual caseins, or associate into a stable casein micelles (112). The characteristics of individual caseins are similar to the characteristics of the molten globule folding intermediate. This folding intermediate contains significant secondary structure but a largely flexible and disordered tertiary structure with exposed hydrophobic regions (13).

*Structural Properties of Caseins: $\alpha_S$-Caseins, $\beta$-Casein, and $\kappa$-Casein*

$\alpha_S$-Caseins ($\alpha_{S_1}$-casein and $\alpha_{S_2}$-casein) have the highest content of phosphate of the caseins and are very sensitive to calcium. Both $\alpha_S$-caseins show strong association and readily precipitate at low pH, or with calcium at neutral pH (19, 87). For $\alpha_{S1}$ casein, it is a single polypeptide chain with 199 amino acid residues and a molecular weight of 23,619 Da. Three-dimensional molecular modeling of $\alpha_{S_1}$-casein shows a hydrophilic amino terminal portion, a segment of hydrophobic $\beta$-sheet, a phosphopeptide segment and a very hydrophobic carboxyl-terminal domain (87). The $\alpha_{S1}$ casein molecule contains eight phosphate residues, all in the form of serine monophosphate. Seven of these phosphoserine residues are clustered in an acidic portion of the molecule bounded by residues 43 and 80 (24). The predicted modeling shows seven of these phosphoserine residues to be located on $\beta$-turns, which is compatible with known phosphorylated residues in crystallized proteins (59). This cluster forms a highly hydrophilic domain on the right shoulder of the molecule and is bounded by prolines 29 and 75. There is a high degree of hydrophobicity in the segment containing residues 100-199 and this region is probably responsible, in part, for the pronounced self-association of the $\alpha_{S_1}$-casein monomer in aqueous solutions. This self–association approaches a limiting size under conditions of lowered ionic strength; the highly charged phosphopeptides region (through

charge repulsions). The hydrophobic C-terminal domain contains two segments of extended β-strands (residues 134 to160 and 163 to 178). These extended β-strands may lead stability to casein polymers through sheet by sheet interactions (59). The carboxyl terminal region is also rich in tyrosine and nitration of $\alpha_{S1}$ casein leads to decreased stability in reconstituted micellar structures. The hydrophobic segment (100-199) represents a hydrophobic domain and contains a segment of non-stranded α-sheet (24). $\alpha_{S_2}$-Casein consists of a large number of positively charged side chains, especially in the C-terminal segment and contains two cystein residues. However, it has a high number of phosphorylations and a low proline content. Two segments, 50-13 and 130 to 207, contain 38% identical residues to $\alpha_{S_2}$-casein. This high internal homology suggests that $\alpha_{S_2}$-casein may be the product of duplication of a primitive gene (19, 50).

The β-casein molecule is a single chain of known sequence with five phosphoserine residues and a molecular weight of 23,980 Da (58). The refined modeling of β-casein shows a loosely packed, asymmetrically structure with an axial ratio of 2:1. Hydrophobic side chains were uniformly dispersed over one end (C terminal) and the center surface of the structure; the other end (N-terminal) was hydrophilic (58). The hydrophobic section also possessed a large loop through which water could easily travel. Such a suprasurfactant structure could account for the micellar type of hydrophobically

driven self-association exhibited by β-casein (50). The N-terminal portion of the β-casein molecule (residues 1 to 40) contains the phosphoserine residues and carries essentially all of the protein's net charge as well as most of the protein's potential α-helical residues (50). The C-terminal one third of the molecule (residues 136 to 209) contains many apolar residues (containing in its high hydrophobicity) and only two short stretches of potential β-structure (58). β-Casein undergoes an endothermic self-association that reaches a maximum or limiting size depending on the ionic strength. The N-terminal concentration of charge, coupled with the highly hydrophobic C-terminal, may account for the temperature dependence of this self-association, because hydrophobic interactions are temperature-dependent (113). The self-association of β-casein can be fitted best to a model that describes the association as proceeding through a critical micelle concentration and achieving a limiting size (50, 113). Like $\alpha_{s1}$ casein, β-casein is insoluble at room temperature (24 °C) in the presence of $Ca^{2+}$ at total calcium concentrations below those encountered in milk. However, precipitation of β-casein from solution is temperature-dependent, and calcium-β-caseinate complex is soluble at 1 °C at concentrations of up to 400 mM $Ca^{2+}$ (58, 113). This temperature dependence is demonstrated in the release of β-casein from the casein micelle at temperatures between 2 and 4 °C (113).

In aqueous solution, β-casein occurs as a random coil with little or no regular secondary structure (58).

For κ-casein, the three dimensional structure modeling shows two unstranded β-sheets; both are predominantly hydrophobic and capable of forming quaternary structural interaction sites with $\alpha_{S1}$ casein (24). In descriptive terms, κ-casein can be thought of as being represented by a "horse and rider." The amino-terminal 110 to 120 residues represent the "horse" and the carboxy-terminal portion represents the " rider." Two distinct legs are seen in the "horse" portion model (60). These legs are generated by β-sheet regions comprising residues 20 to 25; 29; 39 to 45; and 49 to 55, which are connected by γ or β-turns (60). κ-Casein contains two cysteine residues. Whether these can form intra/intermolecular disulfide bonds and the effects of such bonding on micelle stabilization have not been clearly establized. The amino-terminal fifth of the molecule has a relatively high charge frequency; however, the net charge is zero, and this part of protein, although relatively hydrophobic, is somewhat exposed. Residues 20 to 68 represent an exceptionally hydrophobic area with almost no charge (24). It is precisely within this region that the majority of the residues found in the "legged" structures of the κ-casein molecule occur. These nonstranded, highly hydrophobic β-sheet make ideal sites for sheet by sheet interactions with other κ-casein molecules or with hydrophobic

domains of $\alpha_{S1}$ or β-caseins. Indeed, the concentration-dependent reaction profile of the reduced form of purified κ-casein can be fitted with a model for polymerization at a critical micelle concentration of 0.05% (60). κ-Casein differs from other caseins in that it is soluble over a broad range of calcium ion concentrations. It was this calcium solubility that led to assign to it the role of casein micelle stabilization (24). It is also the κ-casein fraction that is most readily cleaved by chymosin (rennin); the resulting products are termed para-κ-casein and the macropeptides. It would appear that κ-casein is the key to micelle structure in that it stabilizes that calcium-insoluble $\alpha_{S1}$ or β-caseins (60, 124). Of the major components of the casein complex, only κ-casein can be glycosylated. Nearly all the carbohydrate, as well as the phosphate associated with κ-casein, is bound to the macropeptide, which is the highly soluble portion liberated by chymosin hydrolysis (60, 124). The major site for glycosylation, Thr-133, is found in the model on the back of the "horse" and is on a β-turn. The sites of phosphorylation, Ser-149 and Thr-145, are on the back portion and are found in β-turns as well (60).

*Interactions Between Milk Proteins and Molecular Chaperones*

The inclusion of milk proteins into chaperone mediated refolding studies began with Martin et al. (71) and Langer et al. (62). Martin et al. (71) investigated the *in vitro*

folding of rhodanese mediated by GroEL, GroES and ATP and proposed that GroEL recognized the folding intermediate described as the molten globule. αs-Casein was used as a competitor protein binding to GroEL since αs-casein exposes a considerable part of its hydrophobic residues to the solvent. αs-Casein was able to bind to GroEL and displace rhodanese from GroEL. αs-Casein was released from GroEL by ATP. Further studies by Langer et al. (62) demonstrated that αs-casein can displace rhodanese from DnaJ, but was released in the presence of ATP. Dessauer and Bartlett (18) used the interaction between casein and GroEL to displace unfolded Rbisco-protein A fusion from GroEL to obtain a pure sample of fusion protein.

Langer et al. (62) showed that DnaK interacts with extended polypeptide chains *in vitro*. CMLA is a derivative of α-lactalbumin which maintains an extended conformation lacking stable secondary structure in the absence of denaturant. DnaK was found to bind CMLA in a temperature dependent manner, with less efficient binding at 25 °C compared to 37°C. The presence of ATP also resulted in dissociation of the DnaK/CMLA complex. Szabo et al. (114) found that the inclusion of CMLA inhibited the DnaK, DnaJ, and GrpE mediated refolding of luciferase by specifically binding DnaJ. Therefore, both DnaJ and GroEL bound αs-casein and could be released in the presence of ATP.

DanK could be released from CMLA by either lowering the temperature or by adding ATP. Casein is also able to displace unfolded proteins from GroEL.

In addition to the major Hsp families (Hsp 70, Hsp 60, and Hsp 90), it is well known that the Clp proteins bind with high affinity to α-casein and degrade it with ATP hydrolysis. Free ClpA was trapped by adding a 10-fold molar excess of α-casein, which binds with high affinity to ClpA (83). ClpB has ATPase which is stimulated 5-10 fold by α-casein (130). Both ClpAP and ClpXP, as ATP dependent proteases, capable of degrading α-casein (99, 119, 125).

## PRACTICAL ASPECTS OF CHAPERONES

The expression of heterologous genes in bacteria is by far the simplest and most inexpensive means available for obtaining large amounts of desired polypeptides for research or commercial purposes (10). However, many heterologous polypeptides expressed in *E. coli* fail to fold into their native state. Instead, they are either degraded by the cellular proteolytic machinery or accumulate in insoluble form, typically as inclusion bodies (30). Therefore, the art of protein folding, or refolding is essential to the biotechnology industry with respect to *in vitro* protein synthesis of recombinant protein expression in a heterologous host (47). Recombinant proteins are currently used as therapeutic aids and enzymes in food processing. Soluble general and specific folding

aids have enhanced the folding of proteins from a denatured state or during protein synthesis by acting as aggregation inhibitors. The general aids (molecular chaperones, sugars, surfactants and polymers) are nonspecific and can be used for many proteins while the specific aids (ligands, inhibitors, substrates and antibodies) are tailored for an individual protein (14, 90, 91). Development of an optimized refolding process for recombinant proteins has been the subject of many publications. Each process was tailored for a specific protein and include the addition of reduced and oxidized thiol reagents, labilizing agents, and ethanol (6, 46).

The optimum folding aid would be cost effective, reusable without reduction in efficiency, inhibit protein aggregation without adversely affecting formation of native protein, easily separated from the native protein after completion of folding and general enough to work for many proteins (14). Molecular chaperones do not change protein folding pathways but increase recovery yields by preventing aggregation side-reactions. Molecular chaperones are found in both eukaryotes and prokaryotes and are involved in protein folding, translocation, translation initiation, gene expression, and growth control (116).

An ideal situation would be to immobilize molecular chaperones along with other general folding aids to enhance protein folding. Buchner et al. (6) showed that DnaK immobilized on BioRad Affi-Gel beads could slightly increase the refolding yields of a

recombinant immunotoxin in the presence of ATP. Similarly, Phadtare et al. (86) found that GroEL immobilized on a matrix derivatized with anti-GroEL antibodies was capable of discharging properly folded tubulin and glutamine synthase upon incubation with GroES and ATP. Monomeric GroEL in *E. coli* retain their ability to bind nucleotides but are unable to suppress the aggregation or promote the refolding of model protein (128). However, truncated versions of the GroEL promotor can function as ATP-dependent chaperone, albeit with low efficiency than the oligometric structure (68, 133). The full-length monomers prepared from the *T. thermophilus* GroEL homolog remain capable of increasing the refolding yields of lactate dehydrogenase and rhodanese in a process that does not require adenonine nucleotides (115). It was further shown that the same monomers facilitate the ATP-dependent refolding of rhodanese when immobilized on a TSK matrix (115). However, immobilized molecular chaperones have shown a lower folding efficiency than the soluble chaperones because of the concerted action exhibited by chaperones and the oligomeric compositions of GroES and GroEL.

## HYPOTHESIS

Molecular chaperones are found in both eukaryotes and prokaryotes and are involved in protein folding, translocation, translation initiation, gene expression, and growth control. They prevent aggregation, assist refolding and mediate degradtion of

misfolded proteins in an ATP dependent manner. Chaperones recognize unfolded polypeptide chains, the molten globule conformation, or hydrophobic sequences. The caseins are amphiphilic proteins with characteristics similar to the molten globule folding intermediate. Therefore, the caseins can be used to affinity purify the *E. coli* molecular chaperones.

**OBJECTIVES**

1. To develop bioselective adsorption matrices for the affinity purification of molecular chaperones from *E. coli*. Both α-casein (combination of $\alpha s_1$-casein and $\alpha s_2$-casein) and β-casein will be immobilized to solid supports.
2. To compare the induction of *E. coli* chaperones with three treatments including heat shock, ethanol stress, and salt stress. Mild and severely stressful conditions will be applied for overexpression of molecular chaperones
3. To determine the interactions and dissociation requirements between immobilized affinity matrices and purified molecular chaperones.
4. To compare source between two *E. coli* strains and column efficiency between affinity matrices (α-casein and β-casein) for the purification of molecular chaperones.

5. To identify the purified molecular chaperones by Western analysis and N-terminal sequencing.

6. To characterize purified molecular chaperones with respect to refolding activity of denatured CAB (carbonic anhydrase B) and ATPase activity.

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# CHAPTER II

## CHARACTERIZATION OF INTERACTIONS BETWEEN *E. COLI* MOLECULAR CHAPERONES AND IMMOBILIZED CASEINS

**Abstract**

The molecular chaperones are a group of proteins that are effective at *in vitro* and *in vivo* protein folding. The molecular chaperones were affinity purified with immobilized α-casein and β-casein columns from two heat induced *E. coli* strains, NM522 and BL21.

Heat induced BL21 cell lysate (10 mg protein) was applied to immobilized α-casein (45 mg/g beads) or β-casein (30 mg/g beads) column. After removing nonspecifically bound proteins with 1 M NaCl, the molecular chaperones were eluted with cold water, 1 mM Mg-ATP, or 6 M urea. The eluates from affinity columns were analyzed by SDS-PAGE. Western analysis identified five *E. coli* molecular chaperones including DnaK, DnaJ, GrpE, GroEL, and GroES in cold water eluates. Among samples, ATP eluates showed the highest chaperone purity of 80-87% followed by cold water eluates with 62-68% purity. The β-casein column showed a higher binding capacity than the α-casein column since β-casein urea eluates contained 3.18 mg total protein of which 58% were chaperones compared to α-casein urea eluates with 2.68 mg total protein of

which 32% were chaperones. For strain comparison, heat treated NM522 cell lysate was also applied to casein columns for the affinity purification of molecular chaperones. NM522 eluates showed more non-chaperone proteins in cold water eluates from both affinity columns.

This research shows that our novel casein affinity chromatography is a rapid and efficient method for the purification of chaperones.

**Introduction**

The art of protein folding or refolding is essential to the biotechnology industry with respect to *in vitro* protein synthesis of recombinant protein expression to successfully produce proteins and enzymes for therapeutic and processing uses.[1)] Molecular chaperones are found in both eukaryotes and prokaryotes. They have roles in many cellular processes including protein folding, translocation, translation initiation, gene expression, and growth control. Furthermore, they also prevent aggregation, assist refolding and mediate degradation of misfolded proteins.[2-4)]

Chaperones may mediate the correct folding and assembly of polypeptides by working in a sequential mechanism on newly synthesized peptides. A model described by Pfanner[5)] shows that DnaJ recognizes and binds newly synthesized peptides or proteins exhibiting no secondary or tertiary structure. DnaK then interacts with the complex to stabilize it. GrpE binds to the DnaK-DnaJ-protein complex and mediates

either the release of the native protein or its transfer to the GroEL/ES complex. The physical interactions between DnaK, DnaJ and GrpE and between GroEL and GroES have been well documented.[6)]

Current methods of purifying chaperones involves overproducing the heat shock transcription factor sigma 32, or inducing the heat-shock response by increasing the cell growth temperature, adding ethanol, or other agents to the growth medium.[7-9)] Several affinity purification methods have been described to purify molecular chaperones from stressed cells.[10-13)] These affinity purification methods showed low recovery and specificity with respect to the *E. coli* molecular chaperones. The immobilized denatured proteins were capable of purifying a small fraction of the molecular chaperones and no one method was successful in purifying more than three different chaperones (8M urea as the eluant). These methods also included procedures that resulted in the release of denatured molecular chaperones. Therefore, immobilized denatured proteins would not be efficient for affinity binding with release of active *E. coli* chaperones.

It has been suggested that chaperones might recognize unfolded polypeptide chains, molten globule conformation, secondary structure or hydrophobic sequences.[5)] Milk caseins are not found individually in milk, but form large quaternary complexes known as casein micelles. The caseins are amphiphilic phosphoproteins with characteristics similar to the molten globule folding intermediate.

Martin *et al.*[14] and Langer *et al.*[15] found that α-CN (α-casein) was used as a competitor protein during the refolding of rhodanese. α-Casein was able to both bind to GroEL and displace rhodanese from GroEL. α-Casein was then released from GroEL by ATP. Langer *et al.*[15] demonstrated that α-CN can displace rhodanese from DnaJ, but was released in the presence of ATP. Dessauer and Bartlett[16] used the interaction between casein and GroEL to displace unfolded Rubisco-protein A fusion protein from GroEL to obtain a pure sample of fusion protein.

The present study developed a novel casein affinity chromatography procedure to purify molecular chaperones from heat stressed *E coli* cells. Five molecular chaperones including DnaK, DnaJ, GrpE, GroEL, and GroES were identified by Western anlysis.

**Material and Methods**

*E. coli Preparation.* Two *Escherichia coli* strains, NM522 (Strategen, Lajolla, CA) and lon$^-$ (lon protease gene deficient) mutant, BL21 (DE 3) (Invitrogen, Carlsbad, CA) were used as the source of heat shock proteins. Cells, 600 ml, were grown to an optical density of 0.6 at 600nm (mid-log phase) at 30°C in NZYM media (Sigma, St. Louis, MO) supplemented with 50 μg/ml ampicillin for NM522 or 34 μg/ml chloramphenicol for BL21. Chaperone expression was induced by the addition of 400 ml media pre-heated to 42°C followed by incubation in a water bath for 1 h. The cells were

harvested by centrifugation for 10 min at 5000 x g. The cell pellets were suspended in 50 mM Tris-Cl (pH 7), 50 mM EDTA, and 1 mM PMSF and lysed by sonication (3x 30s burst with 5 min cooling on ice between bursts) (Cell Disruptor 200, Branson Ultrasonics Corp, Danbury, CT). This sonication step, a modification of the procedure of Evers *et al.*[7] was used to dissociate possible complexes between chaperones and natural target proteins. The supernatants were collected by centrifugation at 10,000 x g for 20 min at 4 °C and vacuum filtered through a 0.45 μm cellulose acetate membrane to completely remove cell debris.

*Casein Immobilization.* Controlled-pore glass (CPG) beads (2000 Å, 20-200 mesh) (Sigma, St. Louis, MO) were derivatized with 3-aminopropyltriethoxy silane and succinylated with succinic anhydride according to Walsh and Swaisgood.[17] Acetic anhydride (10% by vol) in acetone was used to cap excess amino groups. α-Casein and β-casein (Sigma, St. Louis, MO) were immobilized onto succinamidopropyl CPG via carbodiimide. The BCA method (Pierce, Rockford, IL) was used to determine the amount of protein immobilized. A 5-ml or 2-g dry weight column housed the immobilized casein beads to allow circulation of protein solutions and buffers.

*Affinity Purification.* The protein concentration in *E. coli* cell lysates was determined using the BCA kit (Pierce, Rockford, IL). The crude protein solutions (10 mg/ ml) on ice bath were warmed to 37 °C for 2 min before addition to immobilized

casein columns. The casein column (5 ml) was applied with 50 mM Tris-Cl/50 mM EDTA, pH 7 buffer (1 vol) followed by the same buffer containing 1 M NaCl (2 vol) to remove nonspecifically bound proteins. Biospecifically bound proteins were eluted with 1 mM Mg-ATP in water 25 °C (3 vol), water at 4 °C (4 vol), or 6 M Urea at 25 °C (3 vol). The casein column was regenerated with 10% DMSO (2 vol) followed by 50 mM Tris-Cl/ 50 mM EDTA, pH 7 (6 vol). Collected samples were concentrated with a 10 kDa membrane (Amicon, Beverly, MA) for further analysis.

*SDS-PAGE Analysis and Densitometry.* Proteins eluting from the casein columns in various solutions were characterized by 10% acrylamide SDS-PAGE. SDS-PAGE analysis was performed according to Laemmli[18] loading 20 μg protein per lane. Dried gels were analyzed by densitometry to measure the intensities of the protein bands. Based on the amount of protein loaded in each lane, 20 μg, the concentration of the individual protein bands was calculated based on the intensities of the bands. Densitometry was performed on photographic images of dried gels using a scanning laser densitometer and Imager Quant software.

*Western Analysis.* After SDS-PAGE analysis of samples, proteins were electro-transferred onto nitrocellulose membrane as described by Towbin *et al.*[19] The membrane was blocked with 2% BSA with gentle agitation for 1 h at room temperature. The membrane was incubated overnight at 4 °C with antibodies against *E. coli* GrpE, DnaJ,

DnaK (StressGen Biotechnologies Corp, Victoria, Canada), GroEL, or GroES (Sigma, St. Louis, MO). After washing blots in TBS containing 0.1% Tween 20, blots were incubated with either anti-rabbit or anti-mouse antibody-peroxidase conjugate (Sigma, St. Louis, MO) for 1 h at room temperature. After additional washes in TBS buffer, the membranes were developed with 4-chloro-1-napthol.

**Results**

*Chaperone Purification.*

A lon$^-$ mutant BL21 was used as a source of molecular chaperones. Heat shock at 42°C was applied to induce chaperone expression in *E. coli* cells as described by Thomas and Baneyx.[9] Covalent immobilization resulted in 45 mg protein/g beads for α-CN (α-casein) and 30 mg protein/g beads for β-CN (β-casein). Five milliliters of beads (2 g) were used for protein purification. Approximately 10 mg crude cell lysate was added to each column as the starting material. Cold water (4 °C) was applied to elute chaperones from the casein columns since hydrophobic interactions are reduced at low temperatures according to Brunshier *et al.*[20] and Langer *et al.*[15]

SDS-PAGE analysis of proteins eluted from casein columns is shown in Fig. 4. Lane α-CN or β-CN shows proteins eluted with cold water from α-CN or β-CN column after removing nonspecifically absorbed proteins with 1 M NaCl.

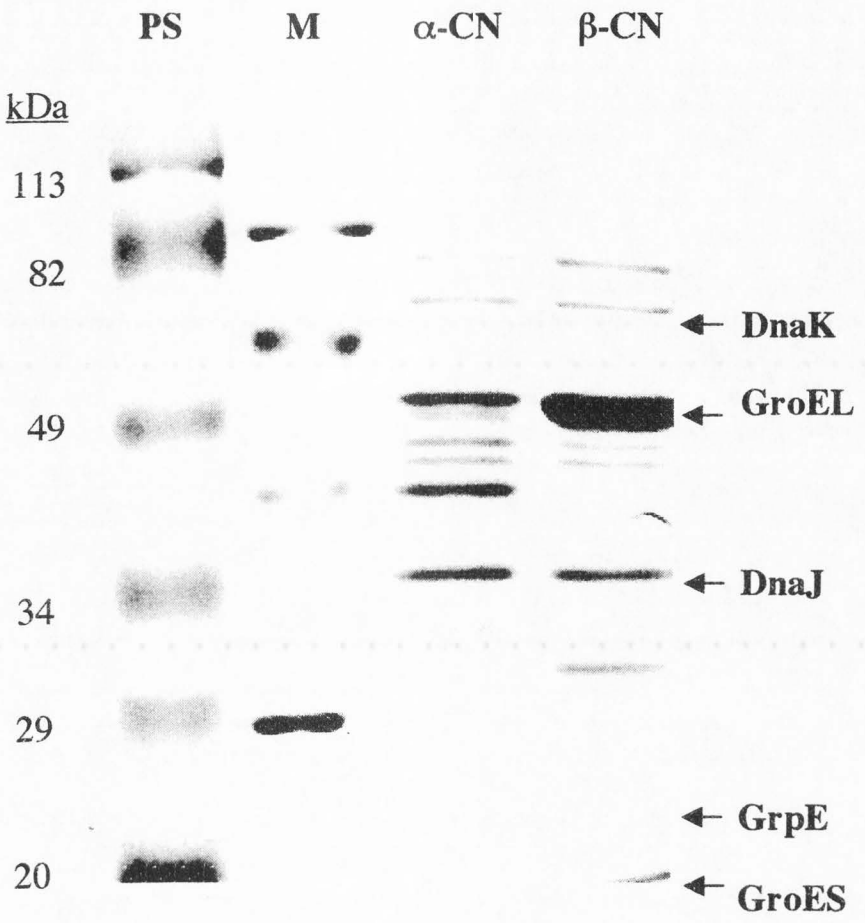


**Fig. 4.** SDS-PAGE analysis of proteins purified from BL21 using α-casein and β-casein columns.

Arrows designate five different proteins with expected molecular weights of DnaK, GroEL, DnaJ, GrpE, and GroES in samples. Lane PS: prestained protein molecular weight marker; lane M: protein molecular weight marker; lane α-CN: proteins eluted using cold water from α-casein column; lane β-CN: proteins eluted using cold water from β-casein column.

β-CN eluates showed less non-chaperone proteins than α-CN eluates as identified in Figs. 5-9. Interestingly, β-CN eluates had a higher concentration of GroEL than α-CN eluates. Although the protein profiles were similar in two columns eluates (Fig. 4), the higher concentration of GroEL in β-CN eluates suggests that GroEL may interact with a higher affinity to β-CN. Major bands had the expected molecular weights of four different *E. coli* chaperones including DnaK (70 kDa), GroEL (57 kDa), DnaJ (41 kDa), and GroES (10 kDa) (Figs. 4-9).

*Chaperone Identification.*

The cold water eluates from the casein columns were analyzed by SDS-PAGE (Fig. 4) and Western blot (Figs. 5-9). DnaK was identified by Western analysis with an antibody against DnaK resulting in a prominent band of 70 kDa (Fig. 5). Protein band, with molecular weights of 57 kDa was identified by Western analysis with antibody against GroEL (Fig. 6). Two bands were recognized by antibodies against DnaJ, both approximately 40 kDa (Fig. 7). The lower band may be due to proteolysis during purification or the antibody used was cross-reactive against another E. coli protein. The presence of GrpE, with molecular weight of 24 kDa, was confirmed by Western analysis with antibody against GrpE (Fig. 8) although it did not appear in the SDS-PAGE analysis (Fig. 4).

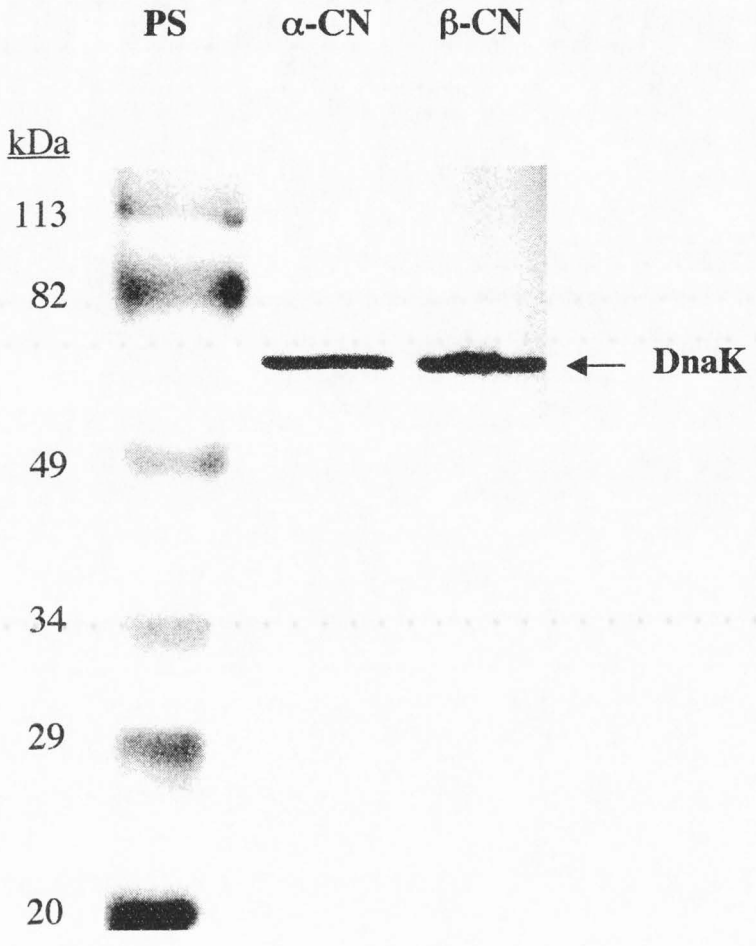


**Fig. 5.** Western analysis of chaperone purified from BL21 using α-casein and β-casein columns.

Lane PS: prestained protein molecular weight marker; lane α-CN: proteins eluted using cold water from α-casein column; lane β-CN: proteins eluted using cold water from β-casein column. The blot shows that DnaK was identified by its corresponding antibody. Arrow designates DnaK with the expected molecular weight of 70 kDa

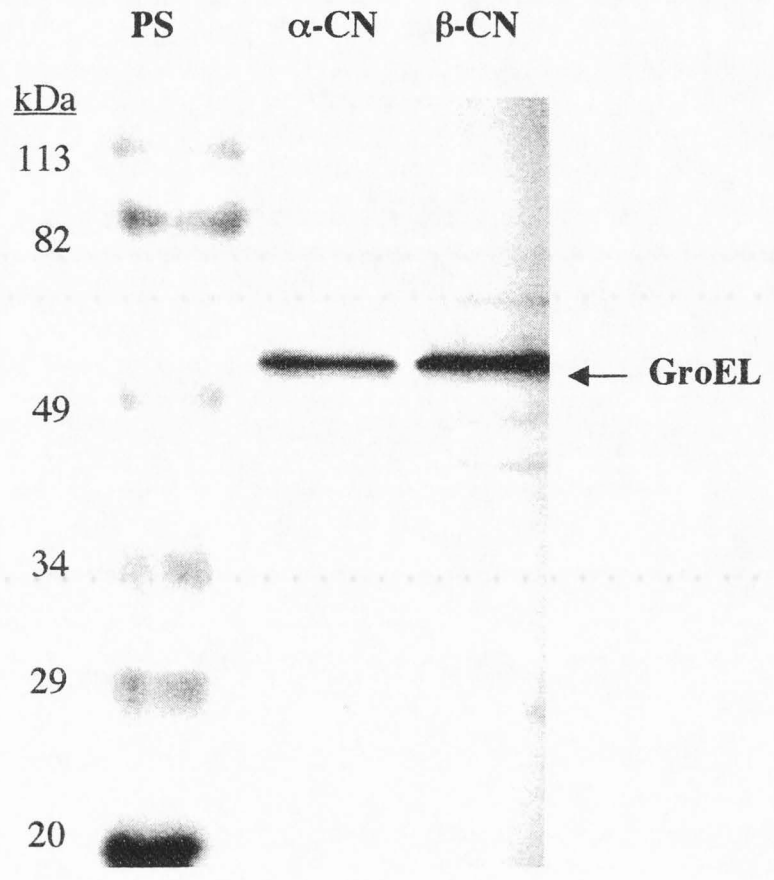


**Fig. 6.** Western analysis of chaperone purified from BL21 using α-casein and β-casein columns.

Lane PS: prestained protein molecular weight marker; lane α-CN: proteins eluted using cold water from α-casein column; lane β-CN: proteins eluted using cold water from β-casein column. The blot shows that GroEL was identified by its corresponding antibody. Arrow designates GroEL with the expected molecular weight of 57 kDa

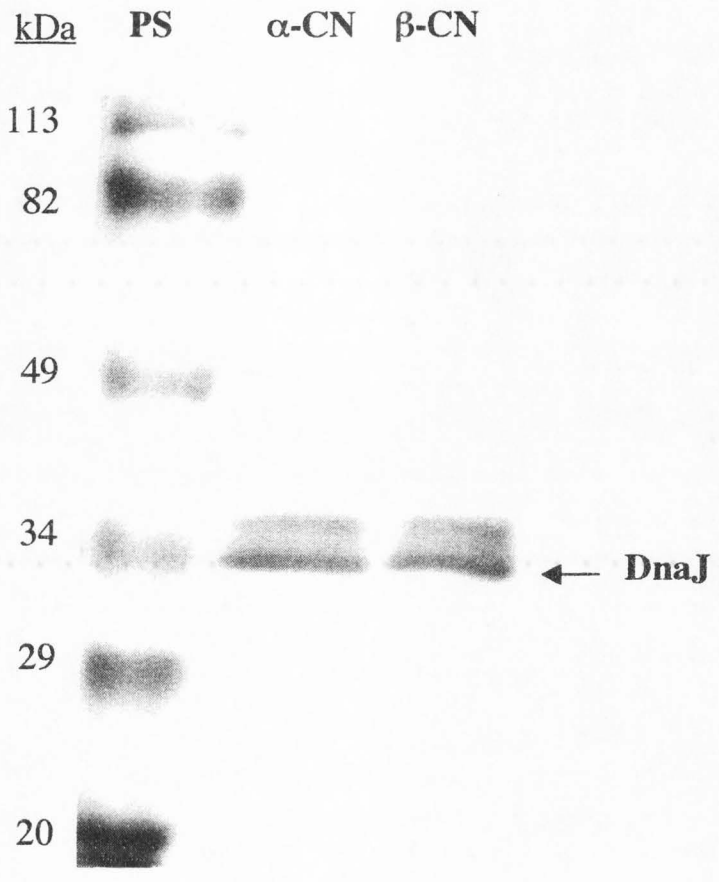


**Fig. 7.** Western analysis of chaperone purified from BL21 using α-casein and β-casein columns.

Lane PS: prestained protein molecular weight marker; lane α-CN: proteins eluted using cold water from α-casein column; lane β-CN: proteins eluted using cold water from β-casein column. The blot shows that DnaJ was identified by its corresponding antibody. Arrow designates DnaJ with the expected molecular weight of 41 kDa

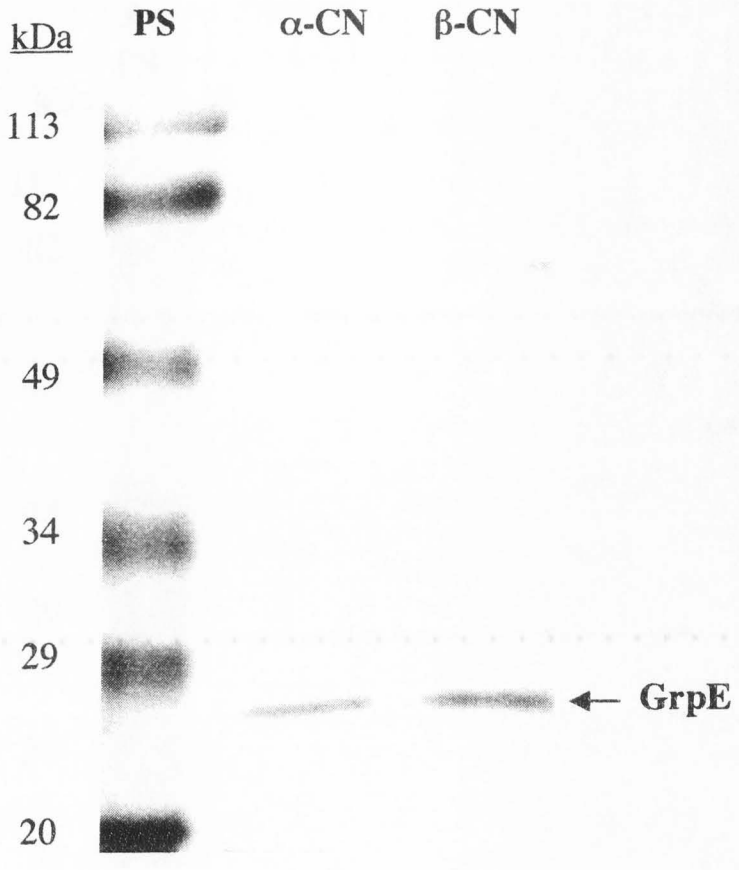


**Fig. 8.** Western analysis of chaperone purified from BL21 using α-casein and β-casein columns.

Lane PS: prestained protein molecular weight marker; lane α-CN: proteins eluted using cold water from α-casein column; lane β-CN: proteins eluted using cold water from β-casein column. The blot shows that GrpE was identified by its corresponding antibody. Arrow designates GrpE with the expected molecular weight of 24 kDa

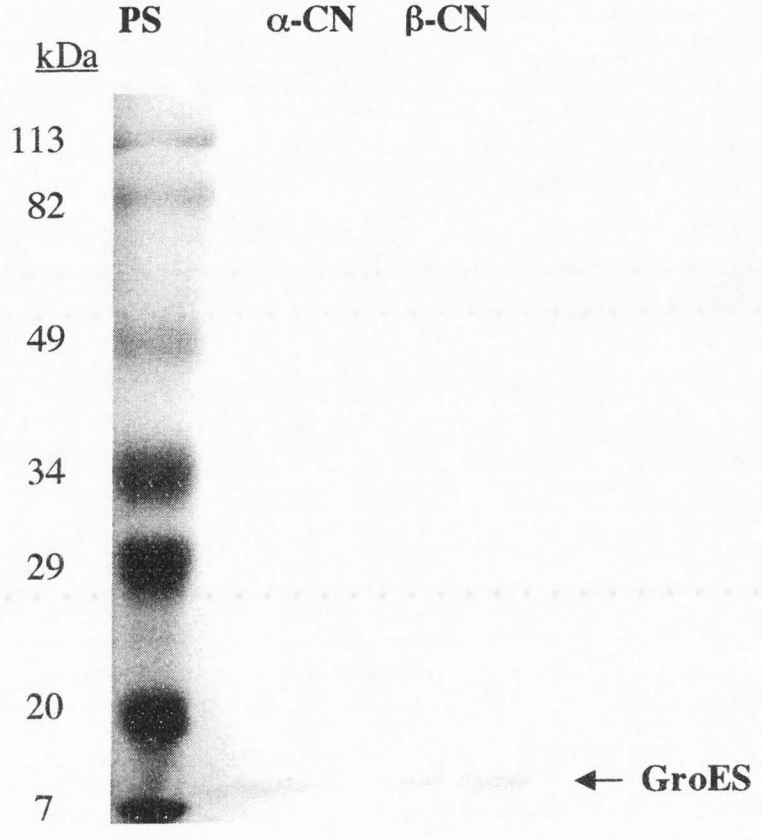


**Fig. 9.** Western analysis of chaperone purified from BL21 using α-casein and β-casein columns.

Lane PS: prestained protein molecular weight marker; lane α-CN: proteins eluted using cold water from α-casein column; lane β-CN: proteins eluted using cold water from β-casein column. The blot shows that GroES was identified by its corresponding antibody. Arrow designates GroES with the expected molecular weight of 10 kDa

One band with a molecular weight of approximately 10 kDa was recognized by an antibody against GroES (Fig. 9). Western analysis results indicate that cold water eluates contained five chaperones including DnaK, DnaJ, GrpE, GroEL, and GroES from α-CN and β-CN affinity columns (Figs. 5-9).

*Interaction between Chaperones and Caseins.*

The E. coli BL21 strain was used as a source of chaperones to investigate elution patterns using three different solutions and two different affinity matrices. The α-CN and β-CN columns were washed with 1 M NaCl in 0.05 M Tris buffer to reduce nonspecifically adsorbed proteins before elution of bound proteins with either: 1) 1 mM Mg-ATP, 2) water at 4°C, or 3) 6 M urea in water. It is assumed that the proteins eluting with 6 M urea represent all bound proteins, while the proteins eluting with water and Mg-ATP represent proteins bound either via hydrophobic or a specific interaction.

Urea eluates showed the highest total protein in eluates compared to 1 mM Mg-ATP or cold water (Table 1). Therefore, Mg-ATP and cold water allowed a specific elution of adsorbed proteins interacting via hydrophobic interaction.

SDS-PAGE analysis of proteins eluting with the various treatments is shown in Figs. 10 and 11. The protein banding profile of samples from the α-CN (Fig. 10) and β-CN (Fig. 11) columns were different.

**Table 1.** Chaperone Elution from α-Casein and β-Casein Columns under the Various Conditions[1]

| | α-Casein column | | β-Casein column | |
|---|---|---|---|---|
| Samples[2] (column volume) | Total protein[3] | Cpns Content[4] | Total protein[3] | Cpns content[4] |
| 0.05 M Tris-Cl, 1 M NaCl (2 vol) | 3.09 ± 0.14 mg | 28.1% (0.87 mg) | 3.16 ± 0.09 mg | 18.2% (0.58 mg) |
| Cold Water at 4 °C (4 vol) | 1.19 ± 0.06 mg | 62.2% (0.74 mg) | 1.21 ± 0.22 mg | 66.4% (0.80 mg) |
| 1 mM Mg-ATP at 25 °C(3 vol) | 0.53 ± 0.01 mg | 80.3% (0.43 mg) | 0.55 ± 0.02 mg | 87.5% (0.48 mg) |
| 6 M Urea at 25 °C(3 vol) | 2.68 ± 0.19 mg | 32.2% (0.86 mg) | 3.18 ± 0.09 mg | 58.0% (1.84 mg) |

[1] A lon⁻ mutant *E. coli* strain, BL21 was used as source of chaperones. A total of 10 mg cell lysate protein was applied to the affinity column.

[2] After applying cell lysate to column, the column washed with 1 M NaCl in 0.05 M Tris-Cl buffer to remove nonspecifically
adsorbed proteins before elution of bound protein with cold water, Mg-ATP, or urea.

[3] Total protein in eluates from the affinity column was determined using the BCA method.

[4] Samples were separated by SDS-PAGE analysis and scanned by densitometry. Chaperone content in samples was detected as % and calculated to actual amount.

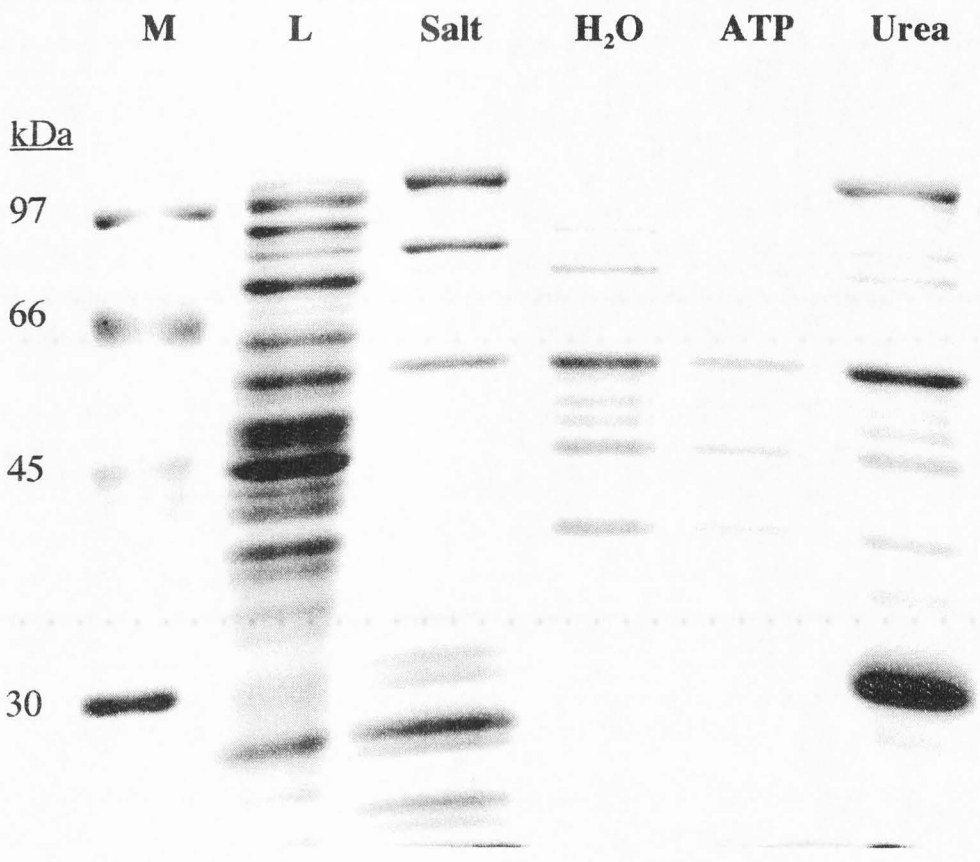


**Fig. 10.** SDS-PAGE analysis of proteins purified from BL21 using α-casein column.

Approximately 20 μg of protein was applied for analysis. Heat shock at 42 °C was used for induction. Lane M: protein molecular weight marker; lane L: crude *E. coli* lysate; lane salt :proteins eluted using 1 M NaCl; lane $H_2O$: proteins eluted using cold water; lane ATP: proteins eluted using 1 mM Mg-ATP; lane Urea: proteins eluted using 6 M urea.

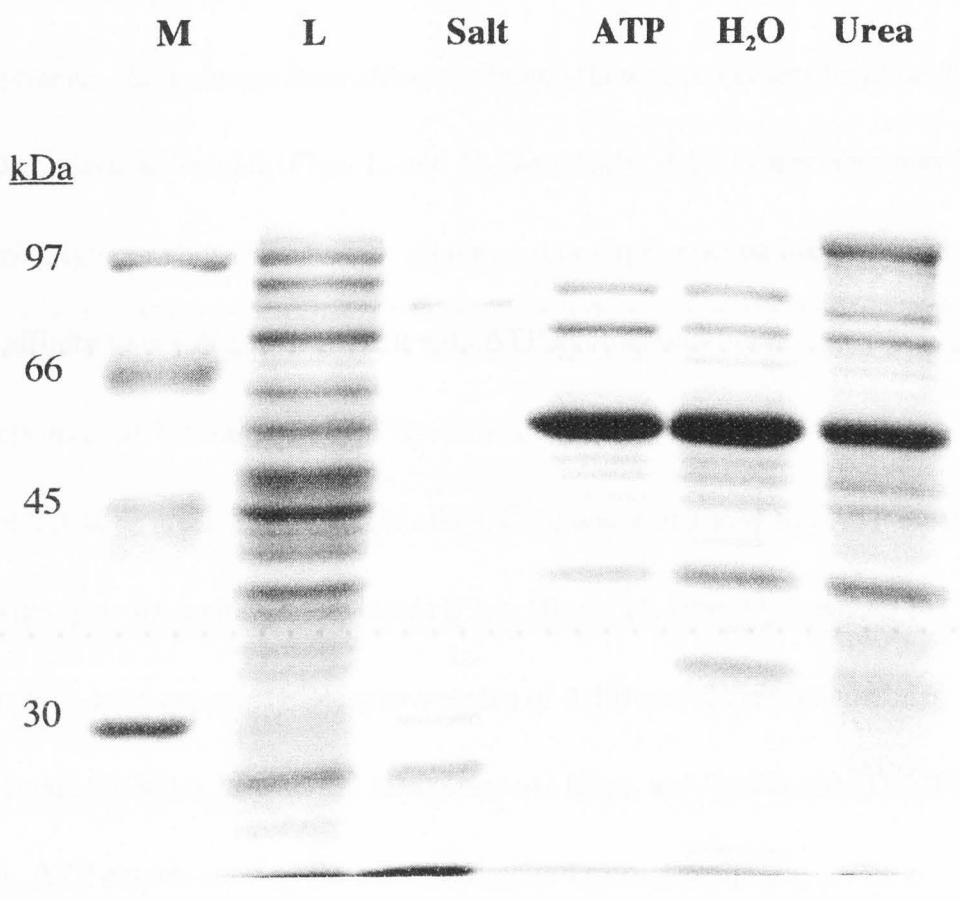


**Fig. 11.** SDS-PAGE analysis of proteins purified from BL21 using β-casein column.

Approximately 20 μg of protein was applied for analysis. Heat shock at 42 °C was used for induction. Lane M: protein molecular weight marker; lane L: crude *E. coli* lysate; lane Salt: proteins eluted using 1 M NaCl; lane ATP: proteins eluted using 1 mM Mg-ATP; lane $H_2O$: proteins eluted using cold water; lane Urea: proteins eluted using 6 M urea.

Before eluting chaperones, washing with 1 M NaCl removed nonspecifically adsorbed proteins. Salt eluates from affinity columns showed prominent bands at 78 kDa and 23 kDa molecular weights (Figs. 10 and 11, lanes salt). A 23 kDa protein may be ClpP (caseino lytic or chaperone linked) protease since ClpP protease are known to bind with high affinity to α-CN and degrade it with ATP hydrolysis.[21)]

Hoskins *et al.*[22)] found that ClpP becomes dissociated from the α-CN in the presence of 0.3 M or greater salt concentration. Cold water or 1 mM Mg-ATP eluates showed as many as six major protein bands (Figs. 10 and 11, lanes $H_2O$ and ATP). These protein bands had the expected molecular weights of 4 different *E. coli* chaperones including DnaK (70 kDa), GroEL (57 kDa) DnaJ (41 kDa), and GroES (10 kDa) (Figs. 10 and 11). ATP eluates showed the least background among samples.

Urea was applied to completely dissociate the bound proteins after washing with 1 M NaCl (Figs. 10 and 11, lanes Urea). Urea eluates display similar protein profiles as seen with cold water with additional protein bands. Urea eluates contained a enriched protein band with molecular weight of 78 kDa which is also shown in salt eluates. However, a 93 kDa protein band in ATP and cold water eluates was not shown in urea eluates.

*Purification Efficiency.*

Chaperone elution from α-CN and β-CN columns under the various conditions is shown in Table 1. The amount of protein loaded onto each column was 10 mg crude cell lysate. The total amount of protein and chaperone content in samples was assessed based on densitomitry scanning of the SDS-PAGE analysis with respect to the concentrations of DnaK, DnaJ, GroEL, and GroES (Figs. 10, 11).

From the α-CN and β-CN columns, the total protein in eluates was 3.1 mg with 1 M NaCl, 1.2 mg with cold water, 0.5 mg with 1 mM Mg-ATP, and 2.7-3.1 mg with 6 M urea, respectively. The high salt washing step (1 M NaCl) to remove nonspecifically bound proteins was efficient since salt eluates contained 3.1 mg total protein of which only 18-28% was chaperone. Specifically bound proteins were eluted with cold water, 1 mM Mg-ATP, or 6 M Urea. Cold water eluates contained 0.7-0.8 mg chaperone or 62%-66% purity. ATP eluates showed the highest chaperone purity of 80-87% among samples yet contained only 0.5 mg protein which is one-third the amount of urea eluates.

Since proteins eluting with 6 M urea represent all bound proteins, the total chaperones in urea eluate was 0.86 mg or 32% purity from α-CN and 1.84 mg or 58% purity from β-CN (Table 1). Therefore, the β-CN column showed a higher binding capacity compared to the α-CN column (Table 1). Considering the total amount of

protein immobilized, the β-CN column binding capacity was 106 μg/mg immobilized protein while the α-CN column resulted in 64 μg/mg immobilized protein

*E. coli NM522 and BL21.*

Two *E. coli* strains, NM522 and lon⁻ mutant BL21 were compared as the source of molecular chaperones. After heat induction of the two strains, molecular chaperones were affinity purified using cold water as eluant from both α- and β-CN columns. The elution profile of proteins from casein columns was analyzed by SDS-PAGE analysis (Fig. 12).

All eluates contained four chaperones plus a 93 kDa unidentified protein as the major bands. NM522 eluates showed more unidentified proteins than BL21 eluates (Fig. 12). BL21 eluates contained more enriched DnaJ protein (41 kDa) than NM522 eluates. Overall, BL21 eluates from β-CN column had the least unidentified proteins among the four elautes.

**Discussion**

Molecular chaperones, belonging to the class heat shock proteins, guide protein folding under physiological condition and prevent irreversible damage of proteins under stress conditions.[5)]

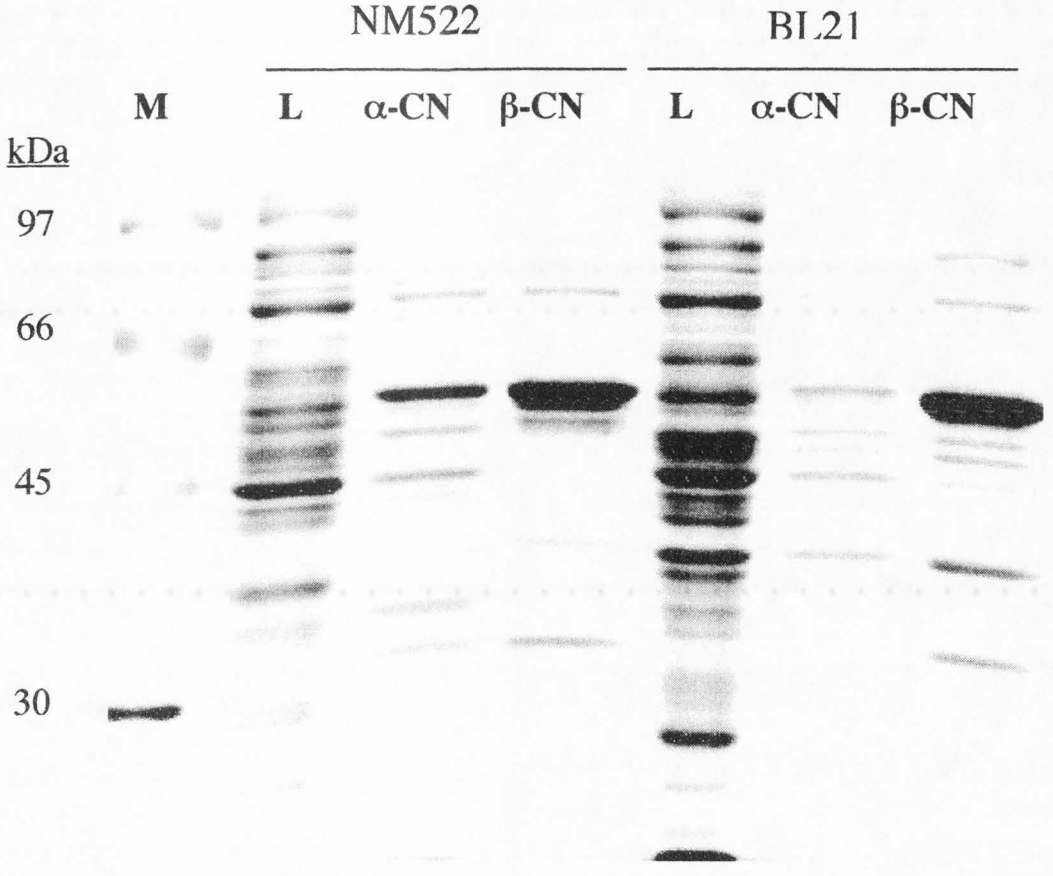


**Fig. 12.** SDS-PAGE analysis of proteins purified from NM522 and BL21 strains using α-casein and β-casein column.

Two *E. coli* strains, NM522 and lon⁻ mutant BL21 were used as the source of chaperone proteins. Approximately 20 μg of protein was applied for analysis. Heat shock at 42 °C was used for induction. Lane M: protein molecular weight marker; lanes L: crude *E. coli* lysates from each strain; lanes α-CN: proteins eluted using cold water from α-casein column; lanes β-CN: proteins eluted using cold water from β-casein column.

The division of labor between the hsp60 and hsp70 groups is not clearly defined. Therefore, DnaK, GroEL, DnaJ, GrpE, and GroES may need to be present or the GroEL/GroES complex may be sufficient for *in vitro* successful protein folding.[6, 23]

Molecular chaperons do not change protein folding pathways but increase recovery yields by preventing aggregation side reaction.[8] In *E. coli*, two molecular chaperone systems, DnaK-DnaJ-GrpE and GroEL-GroES have been studied extensively. DanK alone or the complete DnaK-DnaJ-GrpE system is able to improve refolding yields in the presence of ATP if the substrate size is larger than 65 kDa. GroES is known to bind to GroEL in the presence of ATP and has been shown to form a stable binary complex with polypeptides if the substrate size is smaller than 50-60 kDa.[5, 24] This result could be explained by the relationship between substrate size and chaperone system. Casein is likely to bind to GroEL-GroES chaperones rather than DnaK-DnaJ-GrpE proteins since casein is a small protein with a molecular weight of 24 kDa. This idea is supported by the fact that the 40-70% of total eluted proteins consisted of GroEL and GroES (data not shown).

In comparing column, β-CN had higher binding capacity than the α-CN column, although α-CN (45 mg protein/g beads) showed higher a immobilization efficiency than

β-CN (30 mg protein/g beads). This result indicates that the interactions between chaperones and substrates may be hydrophobic since β-CN is the most hydrophobic protein among milk caseins.[25] Several studies also proved that molecular chaperones bind to protein substrates mainly through hydrophobic interactions. The preferential substrates for DnaK are peptides or unfolded proteins containing internal hydrophobic residues and terminal polar residues. The interactions between GroEL (or GroES) and mMDH (mitochondrial malate dehydrogenase) depend, at least in part, on hydrophobic interactions.[11]

In this study, cold water, 1 mM Mg-ATP, or 6 M urea was used to characterize the interaction and dissociation between immobilized caseins and chaperones. Several studies suggest that the molecular chaperones interact with protein substrates through hydrophobic or ATP-dependent interaction. Brunshier *et al.*[20] and Langer *et al.*[15] demonstrated that GroEL/ES binds to casein *in vitro* via hydrophobic interactions that are influenced by temperature. It was found that Mg-ATP dissociated the complex between chaperones DnaJ, DnaK, GrpE and denatured firefly luciferase according to Szabo *et al.*[26] Urea (6 M) was applied for complete dissociation of the complexes formed according to Evers *et al.*[10]

As the washing step, a high salt concentration (1 M NaCl) reduced nonspecific proteins adsorbed to the column via ionic/electrostatic interactions but did not disrupt the

hydrophobic associations of the chaperones to the immobilized β-CN. Interestingly, associations of the GroEL to the immobilized α-CN are not likely to be specific since all eluates from α-casein column contained the enriched GroEL protein band (Fig. 7). After elution of chaperones with cold water or Mg-ATP, casein column was successfully cleaned and regenerated using DMSO (10%) and the column capacity was maintained for several months. However, 6 M urea, a denaturing agent, decreases the column capacity by 50%.

In this experiment, we compared two *E. coli* strains, NM522 and lon⁻ mutant, BL21 as chaperones sources. Since the lon protease is the major ATP-dependent protease in *E. coli*, casein affinity chromatography could co-purify the lon protease with chaperones.[27] As we expected, eluates from BL21 strain showed less non chaperone protein bands than those from the NM522 strain.

Besides chaperone proteins, several unidentified proteins were enriched in eluates using the casein affinity columns as shown in Figs. 10 and 11. ATP and cold water eluates contained a protein band with the molecular weight of 93 kDa. This protein band may be ClpB protein since ClpB is the only known *E. coli* Hsp100 protein capable of cooperating with DnaK/DnaJ/GrpE.[2, 28] Salt and urea eluates had a prominent protein band with molecular weight of 78 kDa (Figs. 10 and 11).

It was previously shown that the concentrations of intracellular chaperones were increased with induction by heating at 42 °C.[9] The amount of GroEL in *E. coli* cells has been estimated to be 1.6% of the total protein or 2.75 mg/ml which increases 10-fold upon heat shock, reaching up to 12% of total cellular protein.[9] The total amount of protein in cold water eluates was 1.2 mg /10 mg cell lysate, or 12% of the total protein. This is close to the amount expected based on 16% of the total protein consisting of GroEL and GroES.

Purification of GroEL, GroES can include either centrifugation in a sucrose gradient or ammonium sulfate precipitation followed by ion-exchange chromatography,[29, 30] size exclusion,[31] or hydrophobic chromatography.[16, 32] Kubo *et al.*,[33] Szabo *et al.*,[26] and Liberek *et al.*[34] purified three proteins of the Hsp70 family with the combination of several chromatography steps including gel filtration, ion exchange, reverse phase, and affinity techniques. The protein purity was increased by repeated ion-exchange [30, 35] or hydrophobic interaction chromatography.[16, 29, 31-32] Several affinity purification methods have been described to purify chaperones from heat-stress cells in *E. coli*. DnaK, GroEL/ES were affinity purified using Immobilized CRAG,[36] glutathione-S-sepharose,[37] gelatin-agarose,[38] ATP/ADP immobilized agarose,[11] or unfolded polypeptide.[12] DnaJ was affinity purified using celluloase phosphate[33] and Red A agrose.[39] DnaK[40] or Histidine [33] was immobilized for the affinity purification of GrpE.

Although molecular chaperones have been purified and researched extensively in prokaryotes and eukaryotes, current purification procedures do not yield sufficient quantities of active molecular chaperones to investigate the refolding of denature proteins. In this study, we developed caseins affinity chromatography to purify many molecular chaperones in suitable amounts to investigate protein refolding. The casein affinity techniques for purifying chaperones may improve production of recombinant proteins and enzymes for therapeutic and processing uses.

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# CHAPTER III

# AFFINITY PURIFICATION AND CHARACTERIZATION OF THE *E. COLI* MOLECULAR CHAPERONES [1]

## ABSTRACT

The molecular chaperones are a group of proteins that are effective *in vitro* and *in vivo* folding aids and show a well documented affinity for proteins lacking tertiary structure. The molecular chaperones were induced from lon⁻ *E. coli* mutants, affinity purified with an immobilized β-casein column, and assayed for refolding activity with thermally and chemically denatured CAB (carbonc anhydrase B). Chaperones were induced with three treatments; heat shock at 39 °C; heat shock 42 °C; alcohol shock with 3% ethanol (v/v). Lysates were applied to an immobilized β-casein (30 mg/g beads) column. After removing nonspecifically bound proteins with 1 M NaCl, the molecular chaperones were eluted with cold water or 1 mM Mg-ATP. The cold water and Mg-ATP eluates were analyzed by SDS-PAGE. Western analysis identified five *E. coli* molecular chaperones including DnaK, DnaJ, GrpE, GroEL, and GroES. The purity of eluted chaperones was 58% with cold water and 100% with Mg-ATP.

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[1]Reprinted from Seung-Hee Nam and Marie K. Walsh. 2002. Affinity purification and characterization of the *Escherichia coli* molecular chaperones. Protein Express. Purif. 24:282-291

Refolding denatured CAB in the presence of Mg-ATP resulted in a 97% recovery of heat denatured CAB and a 68% recovery of chemically denatured CAB. The use of affinity matrices for the purification which are effective as *in vitro* folding aids will be presented.

## INTRODUCTION

The art of protein folding, or refolding, is essential to the biotechnology industry with respect to *in vivo* protein synthesis of recombinant protein expression in a heterologous host (15). Recombinant proteins are currently used as therapeutic aids and enzymes in food processing. General and specific folding aids have enhanced the folding of proteins from a denatured state or during protein synthesis by suppressing protein aggregation (8, 26). Development of an optimized refolding process for recombinant proteins has been the subject of many publications. Each process was tailored for a specific protein and included the addition of reduced and oxidized thiol reagents, stabilizing agents, and ethanol (6, 14).

The optimum folding aid would be cost effective, reusable without reduction in efficiency, inhibit protein aggregation without adversely affecting formation of native protein, easily separated from the native protein after completion of folding, and general enough to work for many proteins (8). Molecular chaperones are found in both eukaryotes and prokaryotes and are involved in protein folding, translocation, translation

initiation, gene expression, and growth control. An ideal situation would be to immobilize molecular chaperones to enhance protein folding but this may not be possible because of the cascade or concerted action exhibited by chaperones and the oligomeric compositions of GroES and GroEL. However, immobilized DnaK was able to increase slightly the yield of properly folded recombinant immunotoxin (7).

Chaperones may mediate the correct folding and assembly of polypeptides by working in a sequential mechanism on newly synthesized peptides. A model first described by Langer et al. (18) and Martin et al. (19) then modified by Pfanner (24) shows that DnaK, in cooperation with DnaJ, binds to exposed hydrophobic segments of the nascent polypeptide chain or proteins exhibiting little secondary or tertiary structure. DnaJ and GrpE together promote the Mg-ATP-driven reaction cycle of DnaK. Upon its release from DnaK, the polypeptide chain is transferred to the GroEL/ES system where the protein can fold, again with the expense of ATP. A single round or multiple rounds of interaction with these chaperones are required to complete the folding process depending on the protein substrate.

Current methods of purifying chaperones involve overexpression of the chaperones from either heat-stress cells or from a multicopy plasmid in *E. coli*. Several affinity purification methods have been described to purify molecular chaperones from stressed cells (1, 13, 22, 27, 29, 32). These affinity purification methods showed low

recovery and specificity with respect to the *E. coli* molecular chaperones. The denatured proteins or other chaperones immobilized on solid matrix were capable of purifying a small fraction of the molecular chaperones and no one method was successful in purifying more than three different chaperones.

It has been suggested that chaperones might recognize unfolded polypeptide chains, the molten globule conformation, secondary structure or hydrophobic sequences according to Richerme and Kohiyama (25). The interaction between chaperones and proteins is likely to be via hydrophobic unfolded residues in the nascent chains (12). The preferential substrates for DnaK are peptides or unfolded proteins containing internal hydrophobic residues and terminal polar residues. The interactions between GroEL (or GroES) and mMDH (mitochondrial malate dehydrogenase) depend, at least in part, on hydrophobic interactions (12).

Unlike most globular proteins, the milk caseins are not found individually in milk, but form large quaternary complexes known as casein micelles. The caseins are amphiphilic phosphoproteins with characteristics similar to the molten globule folding intermediate. The inclusion of milk proteins into chaperone-mediated refolding studies began with Martin et al. (18) in which casein was used as a competitor protein during the refolding of rhodanese. Caseins were able to both bind to GroEL and displace rhodanese from GroEL. Caseins were then released from GroEL by ATP. Further studies by

Langer et al. (19) demonstrated that caseins can displace rhodanese from DnaJ, but were released in the presence of ATP. Dessauer and Bartlett (9) used the interactions between caseins and GroEL to displace unfolded Rubisco-protein A fusion protein from GroEL to obtain a pure sample of fusion protein.

β-Casein (β-CN) is the most hydrophobic among milk caseins. This protein has a single anionic cluster at the N-terminal region with the remainder being hydrophobic (30). We hypothesize that the large hydrophobic domain of this molecule can interact with chaperones.

The objectives of this research were to affinity purify molecular chaperones from *E. coli* cell lysates using immobilized β-CN. We demonstrate the refolding of carbonic anhydrase B (CAB) with affinity purified molecular chaperones. Five *E. coli* molecular chaperones including DnaK, DnaJ, GrpE, GroEL, and GroES were identified in eluates by Western analysis. Chaperones were included in a refolding study of denatured CAB. The denatured CAB showed 26%-44% of native CAB activity after incubation with purified chaperones which increased to 53-97% with the addition of Mg-ATP.

## MATERIALS AND METHODS

### *Protein Immobilization*

Controlled-pore glass (CPG) beads (2000 Å, 20-200 mesh) (Sigma) were derivatized with 3-aminopropyltriethoxy silane and succinylated with succinic

anhydride according to Walsh and Swaisgood (36). Acetic anhydride (10% by vol) in acetone was used to cap excess amino groups. β-Casein (Sigma) was immobilized on to succinamidopropyl CPG via carbodiimide. The BCA method (Pierce Chemical Co.) was used to determine the amount of protein immobilized. A five-milliliter or 2-g dry weight column housed the immobilized casein beads to allow circulation of protein solutions and buffers.

*Protein Purification and Characterization*

A lon⁻ *E. coli* mutant, BL21 (DE 3) strain with the plasmid plysS (Invitrogen) was used as the source of heat shock proteins. Cells, 600 ml, were grown to a optical density of 0.6 at 600nm (mid-log phase) at 30°C in NZYM media supplemented with 34 μg/ml chloramphenicol. Chaperone expression was induced by the addition of 400 ml media pre-heated to either 39°C or 42°C followed by incubation in a water bath for 1 h. Chaperone expression was induced by the addition of media (25°C) containing 3% ethanol (v/v) followed by incubation at 25°C for 1 h (20, 34). After incubation for 1 h to induce chaperone expression, cells were harvested by centrifugation for 10 min at 5000 x g. The cell pellets were suspended in 50 mM Tris-Cl (pH 7), 50 mM EDTA, and 1 mM PMSF and lysed by sonication (3x 30s burst with 5 min cooling on ice between bursts) (Cell Disruptor 200, Branson Ultrasonics Corp). This sonication step, a modification of

Evers et al. (10), was used to dissociate possible complexes between chaperones and natural target proteins. The supernatants were collected by centrifugation at 10,000 x g for 20 min at 4 °C and vacuum filtered with 0.45 μm cellulose acetate membrane to completely remove cell debris. The protein concentration in lysates was determined using the BCA kit (Pierce Chemical Co.). The crude protein solutions were warmed to 37 °C before addition to an immobilized β-CN column.

The casein column was washed with 50 mM Tris-Cl/50 mM EDTA, pH 7 buffer (1vol) followed by the same buffer containing 1 M NaCl (2 vol) to remove nonspecifically bound proteins. Specifically bound proteins were eluted with 1 mM Mg-ATP in water (3 vol) or water at 4 °C (4 vol). The casein column was regenerated with 10% DMSO (2 vol) followed by 50 mM Tris-Cl/ 50 mM EDTA, pH 7 (6 vol). Samples were concentrated with a 10 kDa membrane (Amicon) for further analysis.

Proteins eluting in 1 mM Mg-ATP or cold water were characterized by 10% acrylamide SDS-PAGE and Western analysis. SDS-PAGE analysis was performed according to Laemmli (17) loading 20 μg protein per lane. Dried gels were analyzed by densitometry to measure the intensities of the protein bands. Based on the amount of protein loaded in each lane, 20 μg, the concentration of the individual protein bands was calculated based on the intensities of the bands. Densitometry was performed on

photographic images of dried gels using a scanning laser densitometer and Imager Quant software.

For Western analysis, proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane (Bio-Rad) and blocked for 1hr in 2% BSA before overnight incubation at 4°C with antibodies against *E. coli* GrpE, DnaJ, DnaK (StressGen Biotechnologies Corp), GroEL, or GroES (Sigma). After washing blots in TBS containing 0.1% Tween 20, blots were incubated with either anti-rabbit or anti-mouse antibody-peroxidase conjugate (Sigma) for 1 h at room temperature. After additional washes in TBS buffer, the membranes were developed with 4-chloro-1-napthol.

*Protein Refolding*

Carbonic anhydrase B (95 μg/ml) was heated at 70 °C for 6 min or denatured in 6 M guanidine-HCl for 24 h. In a refolding experiment, denatured enzyme was incubated with affinity purified chaperones (APCP) or artificial chaperones (ACP) composed of 2.4 mM β-cyclodextrin and 0.2 mM cetyltrimethylammoniumbromide (CTAB) (Sigma). The refolding yield of denatured CAB was measured after the addition of APCP (25 μg), 2 mM Mg-ATP, 1 mM PMSF or α-CN. Phenylmethylsulfonylfluoride (PMSF) or α-CN (250 μg/ml) was used in some studies to prevent ClpP protease activity according to Wojtkowiak et al. (37) and Hoskins et al. (16). The denatured enzyme was diluted into

14 mM Tris-sulfate pH 7.75 containing 10 mM KCl, 2 mM Mg-ATP, and 25 μg of the APCP, for a final concentration of 6.3 μg/ml CAB. For both chemical and thermal denatured CAB, the enzyme activity was measured after 2.5 h incubation at room temperature. CAB activity was monitored via p-nitrophenyl acetate (pNPAc) esterase assay as described by Rozema and Gellman (26). The hydrolysis of pNPAc (4.7 mM final concentration) was measured spectrophotometrically over 60 sec at 400 nm and room temperature. All measurements were normalized to native CAB activity.

## RESULTS

### *Protein Purification*

Covalent immobilization of β-CN resulted in 30 mg protein/g beads. Five milliliters of beads (2 g) was used for chaperone purification. Approximately 10 mg of cell lysate protein was applied to an affinity column as the starting material. Four treatments were used to induce chaperone expression in lon$^-$ *E. coli* mutants; heat shock at 39°C, heat shock 42°C, and alcohol shock with 3% ethanol (v/v) as described by Thomas and Baneyx (34) and Martin et al. (20).

Cold water (4 °C) or 1 mM Mg-ATP in water was applied to dissociate the interactions between β-CN and chaperones since hydrophobic interactions are reduced at low temperatures. The complex between chaperones and denatured firefly luciferase

was dissociated by Mg-ATP (31). SDS-PAGE analysis of proteins eluted with1 mM Mg-ATP or $H_2O$ (4 °C) is shown in Fig. 13. Lane L shows the protein profile of crude cell lysates treated at 39°C (T), 42°C (T), or addition of 3% ethanol (T). Lanes ATP and $H_2O$ show proteins eluted with cold water or Mg-ATP from the β-CN affinity column after removing nonspecifically absorbed proteins with 1 M NaCl. Among the 5–8 bands, the major bands had the expected molecular weights of four different *E. coli* chaperones including DnaK (70 kDa), GroEL (57 kDa), DnaJ (41 kDa), and GroES (10 kDa) (Fig. 13 and 14).

Proteins eluting with Mg-ATP under the three induction treatments showed a similar pattern as cold water eluates with less non-chaperone proteins (data not shown). The cold water eluate of 39°C (T) had the least background among cold water eluates under three different treatments. Although each showed similar patterns, the ratio of chaperones in the cold water eluates were varied (Fig. 14 and Table 2).

*Purification Efficiency*

The purification efficiency of chaperones from β-CN affinity column is shown in Table 2. A total of 10-mg cell lysate protein was applied from each induction treatment to the column. The cold water eluates of 39°C (T), 42°C (T) and 3% ethanol (T) contained 1.69 mg, 1.74 mg, and 1.55 mg of total protein, respectively (Table 2).

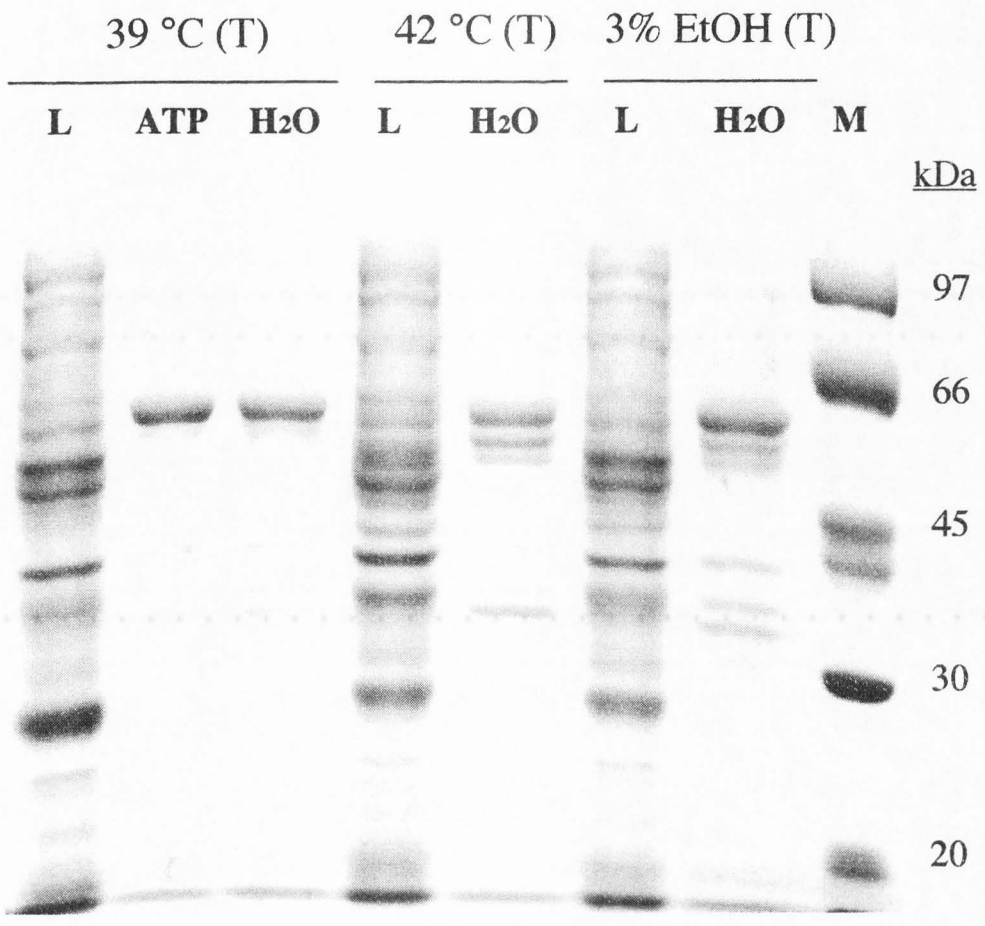


**FIG. 13.** SDS-PAGE analysis of proteins purified from β-casein column. Approximately 20 μg of protein was applied for analysis. Three different treatments (39 °C , 42 °C or 3% ethanol ) were used for induction. Lanes L, crude *E. coli* lysates; Lane ATP, chaperones eluted using 1 mM ATP from 39 °C treated cells. Lanes $H_2O$, chaperones eluted using cold water from cells treated at 39 °C, 42 °C or 3% ethanol. Lane M, protein molecular weight marker.

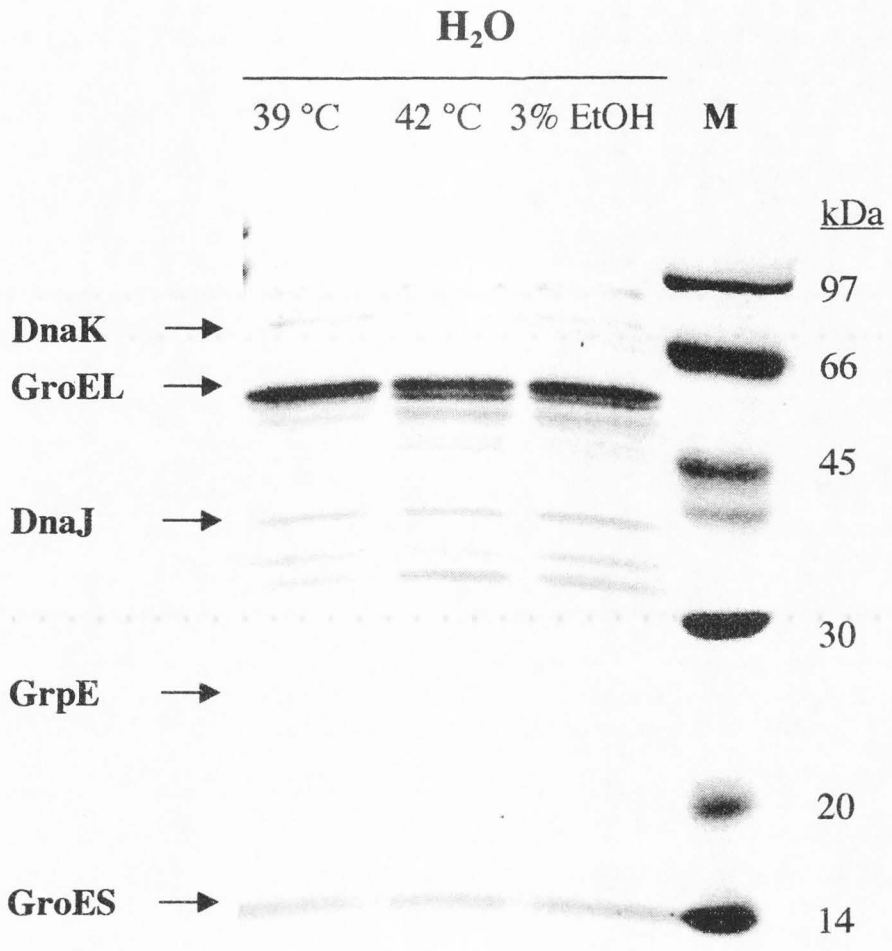


**FIG. 14.** SDS-PAGE analysis of proteins in cold water eluates under three induction treatments. Arrows designate five different proteins with expected molecular weights of DnaK, GroEL, DnaJ, GrpE, and GroES in samples. Lane 3% EtOH, cold water eluate of 3% ethanol treatment; Lane 42 °C, cold water eluate of 42 °C treatment; Lane 39 °C, cold water eluate of 39 °C treatment; Lane M, protein molecular weight marker.

## TABLE 2

Chaperone Elution from β-Casein Affinity Column[1]

| | (ATP elution) | | (Cold water elution) | |
|---|---|---|---|---|
| *E. coli* Treatments | 39 °C [2] | 39 °C [3] | 42 °C [4] | 3% ethanol [5] |
| Total eluted protein[6] | 0.55 ± 0.015 mg | 1.69 ± 0.185 mg | 1.74 ± 0.08 mg | 1.55 ± 0.166 mg |
| DnaK (70 kDa) | 14.4% (0.08 mg) | 6.1 % (0.10 mg) | 8.2% (0.14 mg) | 6.1% (0.10 mg) |
| GroEL(57 kDa) | 49.5% (0.27 mg) | 28.6% (0.48 mg) | 30.1% (0.52 mg) | 20.5% (0.32 mg) |
| DnaJ (41 kDa) | 16.5% (0.09 mg) | 13.6% (0.23 mg) | 8.1% (0.14 mg) | 10.6% (0.16 mg) |
| GrpE (24 kDa) | ND[7] | ND | ND | ND |
| GroES (10 kDa) | 19.6% (0.11 mg) | 10.5% (0.18 mg) | 11.2% (0.20 mg) | 11.7% (0.18 mg) |
| Total Cpns % (mg)[8] | 100.0% (0.55 mg) | 58.8% (0.99 mg) | 57.6% (1.0 mg) | 48.9% (0.76 mg) |

[1]ATP or cold water eluates were scanned by densitometry after SDS-PAGE analysis. Individual protein in eluates was detected as % and calculated to actual amount.

[2]ATP eluate of cells induced with treatment at 39 °C.

[3] Cold water eluate of cells induced with treatment at 39 °C.

[4]Cold water eluate of cells induced with treatment at 42 °C.

[5]Cold water eluate of cells induced with treatment with 3% ethanol.

[6]ATP and cold water eluates under three induction treatments.

[7]Not detected

[8]The total chaperones in eluates as shown as % and amount by combining chaperones.

Overall, cold water eluates of the three treatments showed 50-59% purity based on Densitomity scanning of the SDS-PAGE analysis with respect to the concentration of DnaK, DnaJ, GroEL, and GroES. Mg-ATP eluates at 39 °C(T) showed 100% purity yetcontained only 0.55 mg protein which is one-third the amount of protein eluted with cold water (Table 2).

In *E. coli*, two molecular chaperone systems, DnaK-DnaJ-GrpE and GroEL-GroES have been studied extensively to clarify the protein folding pathway. Thomas et al. (33) suggested that individual chaperones should be present in at least equimolar concentrations for protein refolding. The ratio of chaperones in the eluates under three induction treatments remained relatively constant with respect to the total amounts of the individual proteins as determined by densitometer scanning of SDS-PAGE analysis. For each eluate, GroES was eluted as a 1:2.5 molar complex with GroEL. DanK was eluated as a 1:1 ratio molar complex with DnaJ. However, from the cold water eluate of 39 °C (T), DnaK was eluted as a 1:2 molar complex with DnaJ.

The complex pattern between eluted chaperones conforms with our expectations that GroES, a heptameric ring, would form the 1:2 molar complex with GroEL, a tetradecamer and DnaK would elute as a 1:2 molar complex with DnaJ. GrpE was not detected by SDS-PAGE analysis (Fig. 14). However, Western analysis, which is more

sensitive than SDS-PAGE, proved that GrpE also was eluted from β-CN affinity column (Fig. 18). We assume the absence of GrpE in the SDS-PAGE is due to the detection limits of this analysis.

*Identification of Purified Proteins*

The cold water eluates under three induction treatments were analyzed by SDS-PAGE (Fig. 14) and Western blot (Figs. 15-19). DnaK was identified by Western analysis with an antibody against DnaK resulting in a prominant band of 70 kDa (Fig. 15). Protein bands, with molecular weights of 57 kDa and 41 kDa, were identified by Western analysis with antibodies against GroEL (Fig. 16) and DnaJ (Fig. 17), respectively. The presence of GrpE, with molecular weight of 24 kDa, was confirmed by Western analysis with antibody against GrpE although it did not appear in the SDS-PAGE analysis (Fig. 18). Two bands with molecular weights of approximately 10 kDa and 30 kDa were recognized by an antibody against GroES (Fig. 19). GroES is a heptameric ring of identical 10 kDa subunits. Thus, a 30 kDa band could be a trimer that did not fully dissociated to monomers or the antibody used was cross-reactive against another *E. coli* protein. Western analysis result indicates that Mg-ATP (data not shown) as well as cold water eluted five chaperones including DnaK, DnaJ, GrpE, GroEL, and GroES from β-CN affinity column (Figs. 15-19).

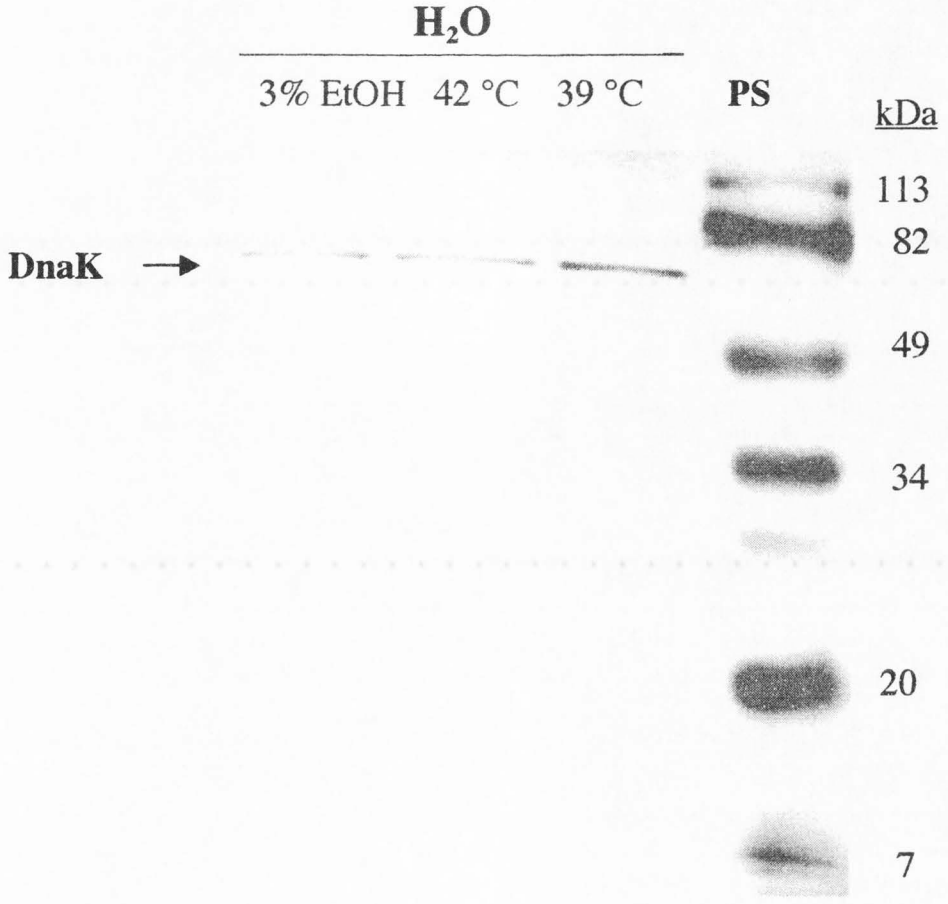


**FIG. 15.** Western analysis of affinity purified chaperone (APCP) in cold water eluates under three induction treatments. Lane 3% EtOH, cold water eluate of 3% ethanol treatment; Lane 42 °C, cold water eluate of 42 °C treatment; Lane 39 °C, cold water eluate of 39 °C treatment; Lane PS, prestained protein molecular weight marker. The blot show that DnaK was identified by its corresponding antibody. Arrow designates DnaK with the expected molecular weight of 70 kDa.

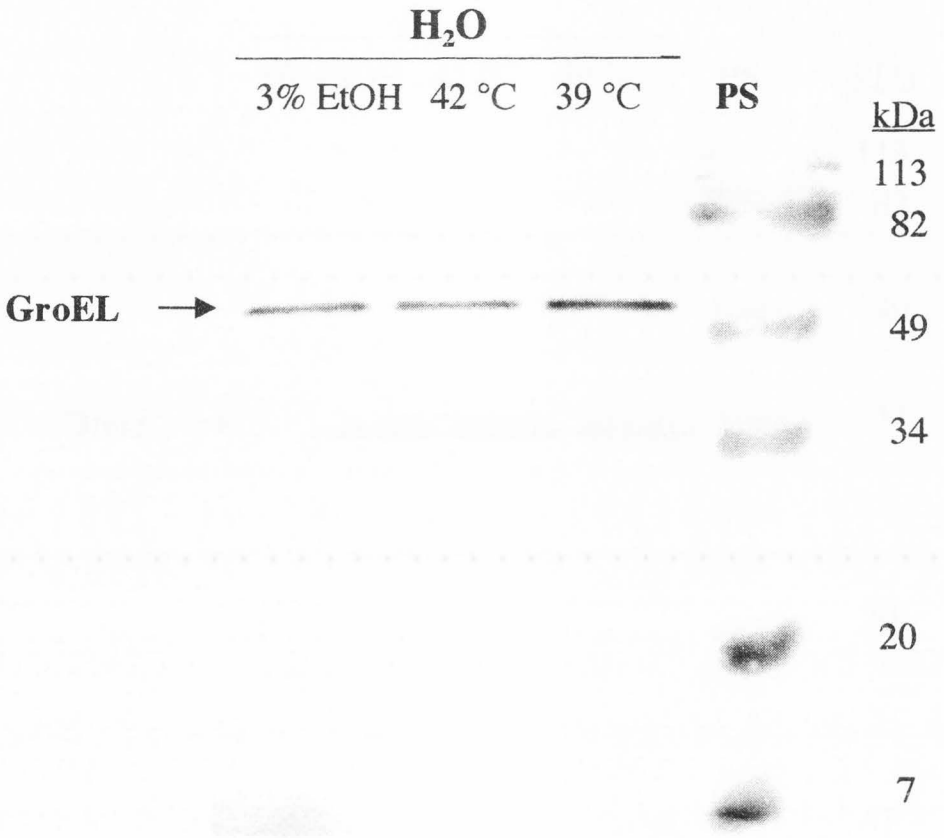


**FIG. 16.** Western analysis of affinity purified chaperone (APCP) in cold water eluates under three induction treatments. Lane 3% EtOH, cold water eluate of 3% ethanol treatment; Lane 42 °C, cold water eluate of 42 °C treatment; Lane 39 °C, cold water eluate of 39 °C treatment; Lane PS, prestained protein molecular weight marker. The blot show that GroEL was identified by its corresponding antibody. Arrow designates GroEL with the expected molecular weight of 57 kDa.

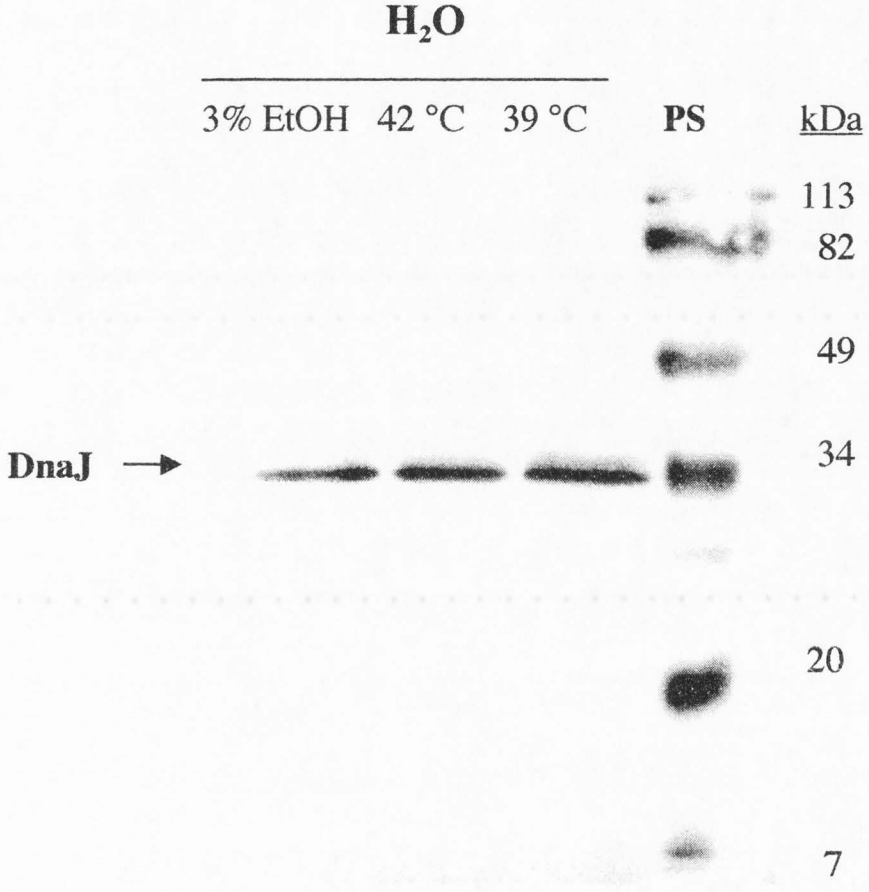


**FIG. 17.** Western analysis of affinity purified chaperone (APCP) in cold water eluates under three induction treatments. Lane 3% EtOH, cold water eluate of 3% ethanol treatment; Lane 42 °C, cold water eluate of 42 °C treatment; Lane 39 °C, cold water eluate of 39 °C treatment; Lane PS, prestained protein molecular weight marker. The blot show that DnaJ was identified by its corresponding antibody. Arrow designates DnaJ with the expected molecular weight of 41 kDa.

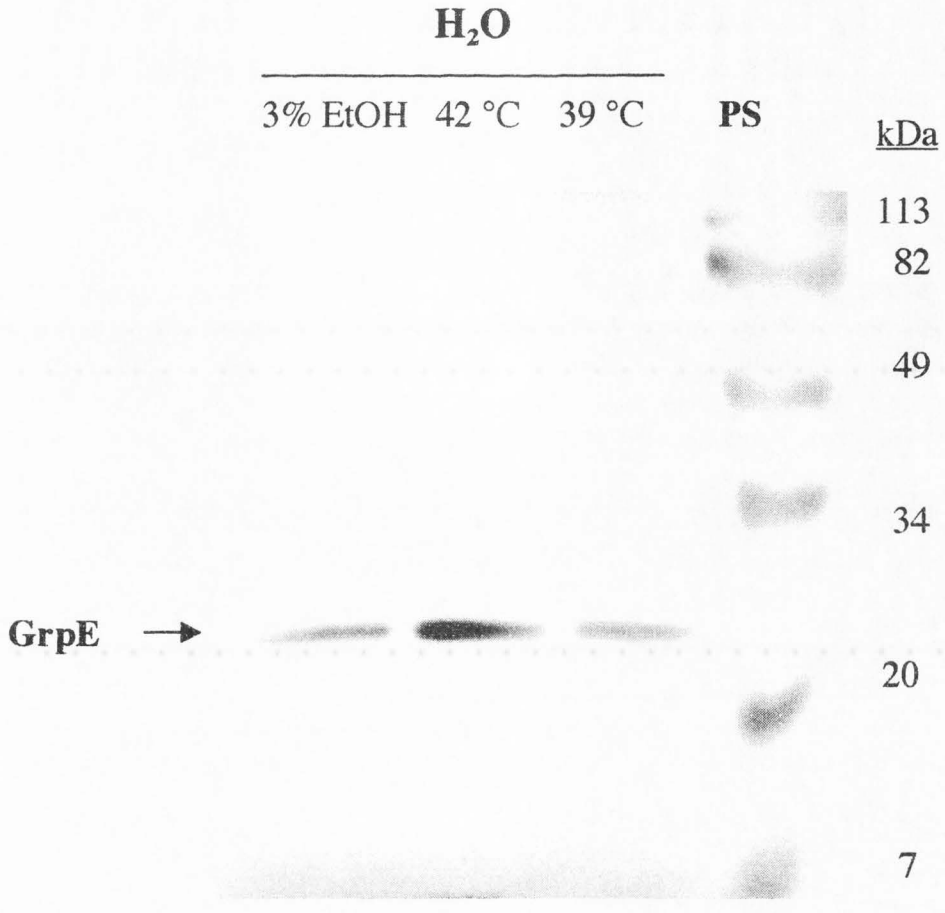


**FIG. 18.** Western analysis of affinity purified chaperone (APCP) in cold water eluates under three induction treatments. Lane 3% EtOH, cold water eluate of 3% ethanol treatment; Lane 42 °C, cold water eluate of 42 °C treatment; Lane 39 °C, cold water eluate of 39 °C treatment; Lane PS, prestained protein molecular weight marker. The blot show that GrpE was identified by its corresponding antibody. Arrow designates GrpE with the expected molecular weight of 24 kDa.

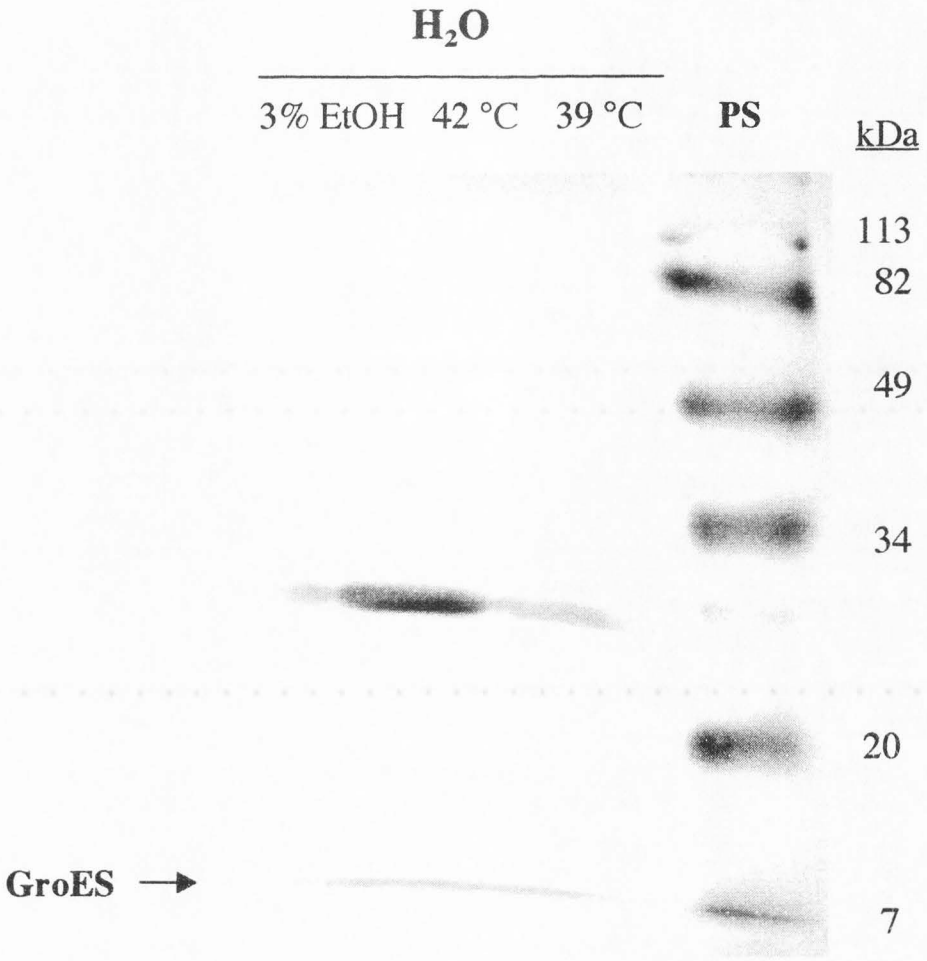


**FIG. 19.** Western analysis of affinity purified chaperone (APCP) in cold water eluates under three induction treatments. Lane 3% EtOH, cold water eluate of 3% ethanol treatment; Lane 42 °C, cold water eluate of 42 °C treatment; Lane 39 °C, cold water eluate of 39 °C treatment; Lane PS, prestained protein molecular weight marker. The blot show that GroES was identified by its corresponding antibody. Arrow designates GroES with the expected molecular weight of 10 kDa.

*Enzyme Refolding Activity by Chaperones*

The refolding activity of CAB using both APCP (affinity purified chaperones) or ACP (artificial chaperones) was monitored spectrophotometrically according to Rozema and Gellman (26) (Tables 3 and 4). In addition to native CAB (N), the denatured CAB and denatured CAB with ACP were included as controls. With heating, the denatured CAB (H) retained 21% of native activity and showed 116% of native activity after incubation with ACP (Table 3). With chemical denaturation, CAB (C) retained 16% of native activity and showed 81% of native activity after incubation with ACP (Table 4). This result indicates that chemical treatment was more efficient at denaturing CAB than was thermal.

ATP (2 mM Mg-ATP) was added to the refolding mixture containing APCP and denatured CAB since chaperone mediated refolding requires ATP (9). The CAB (H) + APCP showed 37% to 44% of native activity. With the addition of 2 mM Mg-ATP, the CAB (H) showed 91% of native activity with APCP eluted with Mg-ATP and 75%-97% of native activity with APCP eluted with cold water (Table 3). On the other hand, the CAB (C) + APCP showed 26% to 38% of native activity.

With the addition of 2 mM Mg-ATP, the CAB (C) showed 69% of native activity with APCP eluted with Mg-ATP and 53-56% of native activity with APCP eluted with cold water (Table 4).

**TABLE 3**

Refolding Activity of Thermally Denatured Carbonic Anhydrase B

| Controls[1] | Activity Mean ± SE[2] | Activity Relative Rate (%)[3] |
|---|---|---|
| CAB (N) | 0.398 ± 0.015 | 100.00 |
| CAB (H) | 0.087 ± 0.001 | 21.96 |
| CAB (H)+ ACP | 0.464 ± 0.006 | 116.71 |
| **Samples [4]** | **Mean ± SE** | **Relative rate (%)** |
| 1. 39 °C (T) ATP | | |
| CAB (H)+ APCP | 0.158 ± 0.02 | 39.80 |
| CAB (H)+ APCP /ATP | 0.363 ± 0.029 | 91.22 |
| CAB (H)+ APCP /ATP (1mM PMSF)[5] | 0.323 ± 0.001 | 81.18 |
| CAB (H)+ APCP /ATP (α-CN)[6] | 0.318 ± 0.005 | 79.96 |
| 2. 39 °C (T) $H_2O$ | | |
| CAB (H)+ APCP | 0.175 ± 0.02 | 44.09 |
| CAB (H)+ APCP /ATP | 0.300 ± 0.029 | 75.32 |
| CAB (H)+ APCP /ATP (1mM PMSF) | 0.378 ± 0.001 | 94.98 |
| CAB (H)+ APCP /ATP (α-CN) | 0.255 ± 0.005 | 64.21 |
| 3. 42 °C (T) $H_2O$ | | |
| CAB (H)+ APCP | 0.147 ± 0 | 37.01 |
| CAB (H)+ APCP /ATP | 0.368 ± 0.002 | 92.49 |
| CAB (H)+ APCP /ATP (1mM PMSF) | 0.391 ± 0.005 | 98.36 |
| CAB (H)+ APCP /ATP (α-CN) | 0.268 ± 0.051 | 67.33 |
| 4. 3% EtOH (T) $H_2O$ | | |
| CAB (H)+ APCP | 0.165 ± 0.01 | 41.59 |
| CAB (H)+ APCP /ATP | 0.387 ± 0.005 | 97.25 |
| CAB (H)+ APCP /ATP (1mM PMSF) | 0.326 ± 0.006 | 81.86 |
| CAB (H)+ APCP /ATP (α-CN) | 0.371 ± 0.004 | 93.26 |

[1]Native enzyme, CAB (N) was denatured by heating for 6 min at 70 °C (H). Denatured enzyme was incubated with artificial chaperone (ACP), the mixture of 2.4 mM β-cyclodextrin and 0.2 mM CTAB.

[2]Enzyme activity was measured in triplication and shown as mean ± standard error.

[3]Relative rate of each sample was calculated on the basis of native enzyme activity as 100% of native.

[4]Samples were ATP or cold water eluates under three induction treatments.

[5]Denatured enzyme (CAB (H)) was incubated with 25 μg affinity purified chaperones (APCP), 2 mM ATP and 1 mM PMSF for 2.5 h at room temperature.

[6]Denatured enzyme (CAB (H)) was incubated with 25 μg APCP, 2 mM ATP and 250 μg α-CN for 2.5 h at room temperature.

**TABLE 4**

Refolding Activity of Chemically Denatured Carbonic Anhydrase B

| | Activity | Activity |
|---|---|---|
| Controls[1] | Mean ± SE[2] | Relative Rate (%)[3] |
| CAB (N) | 0.398 ± 0.015 | 100.00 |
| CAB (C) | 0.066 ± 0.001 | 16.49 |
| CAB (C)+ ACP | 0.322 ± 0.009 | 81.01 |
| Samples [4] | Mean ± SE | Relative rate (%) |
| 1. 39 °C (T) ATP | | |
| CAB (C)+ APCP | 0.152 ± 0.006 | 38.29 |
| CAB (C)+ APCP /ATP | 0.273 ± 0.016 | 68.54 |
| CAB (C)+ APCP /ATP (1mM PMSF)[5] | 0.260 ± 0.002 | 65.45 |
| CAB (C)+ APCP /ATP (α-CN)[6] | 0.208 ± 0.001 | 52.21 |
| 2. 39 °C (T) $H_2O$ | | |
| CAB (C)+ APCP | 0.121 ± 0.012 | 30.31 |
| CAB (C)+ APCP /ATP | 0.210 ± 0.003 | 52.75 |
| CAB (C)+ APCP /ATP (1mM PMSF) | 0.266 ± 0.015 | 66.78 |
| CAB (C)+ APCP /ATP (α-CN) | 0.197 ± 0.007 | 49.43 |
| 3. 42 °C (T) $H_2O$ | | |
| CAB (C)+ APCP | 0.116 ± 0.005 | 29.27 |
| CAB (C)+ APCP /ATP | 0.224 ± 0.011 | 56.18 |
| CAB (C)+ APCP /ATP (1mM PMSF) | 0.240 ± 0.006 | 60.41 |
| CAB (C)+ APCP /ATP (α-CN) | 0.167 ± 0.03 | 41.89 |
| 4. 3% EtOH (T) $H_2O$ | | |
| CAB (C)+ APCP | 0.105 ± 0.001 | 26.45 |
| CAB (C)+ APCP /ATP | 0.224 ± 0.012 | 56.37 |
| CAB (C)+ APCP /ATP (1mM PMSF) | 0.273 ± 0.013 | 68.57 |
| CAB (C)+ APCP /ATP (α-CN) | 0.201 ± 0.002 | 50.61 |

[1]Native enzyme, CAB (N) was denatured by incubation with 6 M Guanidine-HCl for 24 hrs (C). Denatured enzyme was incubated with artificial chaperone (ACP), the mixture of 2.4 mM β-cyclodextrin and 0.2 mM CTAB.

[2]Enzyme activity was measured in triplication and shown as mean ± standard error.

[3]Relative rate of each sample was calculated on the basis of native enzyme activity as 100% of native.

[4]Samples were ATP or cold water eluates under three induction treatments.

[5]Denatured enzyme (CAB (C)) was incubated with 25 μg affinity purified chaperones (APCP), 2 mM ATP and 1 mM PMSF for 2.5 h at room temperature.

[6]Denatured enzyme (CAB (C)) was incubated with 25 μg APCP, 2 mM ATP and 250 μg α-CN for 2.5 h at room temperature.

These results indicate that the CAB (H) and (C) showed an increased activity after incubation with APCP, which was increased further with the addition of Mg-ATP.

Artificial chaperones (ACP) were compared o APCP on their refolding abilities. The CAB (H) showed 117% of native activity with ACP and a range of 75 to 97% of native activity with APCP (plus Mg-ATP) depending on the cell treatments and the eluate. The CAB (C) showed 81% of native activity with ACP and a range of 53 to 69% of native activity with APCP (plus Mg-ATP) depending on the cell treatments and the eluate. It is concluded that APCP is effective for protein folding with a maximum of 97% activity obtained, yet a higher refolding activity was obtained with ACP for the refolding of CAB.

Folding activity was investigated in the presence of the protease inhibitor, PMSF. The ATP-dependent ClpP protease of *E. coli*, with molecular weight of 21 kDa, may be one of the proteases present in purified samples (23). Also, since α-CN is known to bind with ClpP protease, it was added to prevent potential ClpP proteolytic activity (35, 37). PMSF increased APCP activity of cold water eluates (except for sample 4 in Table 3) but did not increase APCP activity of Mg-ATP eluates (samples 1 in Table 3 and 4). This finding suggests that the Mg-ATP eluate does not contain a serine protease. The CAB (H) or (C) + APCP + Mg-ATP showed a 4-20% increase in activity by the addition of 1 mM PMSF. Therefore, PMSF was adequate to inhibit proteases present, including ClpP

protease. However, α-CN did not increase the refolding yield of CAB (H) or (C) by incubation with APCP and Mg-ATP (Tables 3 and 4). From this data, we could conclude that α-CN is not efficient as a protease inhibitor.

## DISCUSSION

Many experiments have documented that chaperones play a critical role in both *in vitro* and *in vivo* protein folding. Since the division of labor between the hsp 60 and hsp 70 groups is not clearly defined, the presence of the GroEL/GroES complex may be sufficient to refold denatured proteins. In order to mimic protein folding *in vitro* for successful folding of denatured or newly synthesized proteins, DnaK, GroEL, DnaJ, GrpE, and GroES may need to be present. Simultaneous overexpression of chaperones in *E. coli* along with a recombinant protein will lead to a decreased level of desired protein, decreased cell growth, and may inhibit proper folding (8).

The methods previously described to purify molecular chaperones would not be efficient for affinity binding and release of active chaperones due to the eluate used (8 M urea), low capture yields, or molecular chaperone binding selectivity. In this study, 1 mM Mg-ATP or water at 4 °C was used to dissociate the interaction between β-CN and chaperones. Several refolding studies proved that the molecular chaperones bind to protein substrates mainly through hydrophobic interactions. Dessauer and Bartlett (9)

and Bhakuni (2) revealed that GroEL/ES binds to casein *in vitro* by a combination of overall hydrophobicity and their hydrophobic interactions are influenced by temperature. The complex of GroEL and tailspike polypeptide dissociated without the addition of MgATP when cooled to 25 °C (4). It was found that MgATP dissociated the complex between chaperones DnaJ, DnaK, GrpE and denatured firefly luciferase according to Szabo et al. (31).

As artificial chaperones, detergents and cyclodextrins have been utilized to duplicate the two-step mechanism of action of the GroEL/ES system in the refolding of CAB (26). In the first step of this artificial chaperone strategy, a detergent (CTAB) captures the nonnative protein upon dilution from denaturing conditions. This process is analogous to the trapping of nonnative proteins within the central cavity of the GroEL. In the second step, the detergent is stripped by addition of β-cyclodextrin to allow productive folding to take place. This is superficially similar to the effect of interactions between GroEL-polypeptides complexes and ATP/GroES that stimulate the release of bound proteins to permit their correct isomerization (33).

In this study, the high salt concentration (1 M NaCl) effectively removed nonspecific proteins adsorbed to the column via ionic/electrostatic interactions but did not reduce the hydrophobic associations of the chaperones to the immobilized β-CN.

After elution of chaperones with cold water or Mg-ATP, the β-CN column was successfully cleaned and regenerated using DMSO (10%) and the column capacity was maintained for several months.

It was previously shown that the concentrations of intracellular chaperones were increased with induction by heating at 39 °C or 42 °C or by addition of 3% ethanol (v/v) to the growth medium (34). The amount of GroEL in *E. coli* cells has been estimated to be 1.6% of the total protein or 2.75 mg/ml which increases 10-fold upon heat shock, reaching up to 12% of total cellular protein (34). The total amount of protein in cold water eluates was 1.5 to1.7 mg/10 mg cell lysate, or 15 and 17% of the total protein. This is close to the amount expected based on 16% of the total protein consisting of GroEL and GroES.

Purification of GroEL, GroES and/or DnaK can include either centrifugation in a sucrose gradient or ammonium sulfate precipitation followed by ion-exchange chromatography (3), size exclusion (5), or hydrophobic chromatography (13). Several affinity purification methods have been developed to obtain chaperones from either heat-stress cells or from a multicopy plasmid in *E. coli*. DnaK, GroEL/ES or Hsp90 were affinity purified using Immobilized CRAG (28), glutathione-S-sepharose (29), gelatin-agarose (22), ATP/ADP immobilized agarose (13), or unfolded polypeptide (27).

Since molecular chaperons do not change protein folding pathways but increase recovery yields by preventing aggregation side reaction, it is clear that they should be present in the refolding buffer in at least equimolar concentrations before addition of denatured substrate (33). In *E. coli*, two molecular chaperone systems, DnaK-DnaJ-GrpE and GroEL-GroES have been studied extensively. DanK alone or the complete DnaK-DnaJ-GrpE system is able to improve refolding yields in the presence of ATP if the substrate size is larger than 65 kDa. GroES is known to bind to GroEL in the presence of ATP and has been shown to form a stable binary complex with polypeptides if the substrate size is smaller than 50-60 kDa (11, 24). Therefore, when a substrate is larger than 65 kDa, DnaK, a monomer, could form the 1:1 molar complex with GrpE, a monomer and the 1:2 molar complex with DnaJ, a dimer. For a substrate smaller than 50-60 kDa, GroES, a heptamer, could bind to GroEL, a tetradecamer by formation of the 1:2 molar complex. In this study, GroES was eluted at a 1:2.5 molar ratio with GroEL from β-CN affinity column, which was expected. The cold water eluate of 39°C only showed 1:2 ratio but the other eluates had equal molar ratio between DnaK and DnaJ. This unexpected result could be explained by the relationship between substrate size and chaperone system. β-Casein is likely to bind to GroEL-GroES chaperones rather than DnaK-DnaJ-GrpE proteins since β-CN is a small protein with molecular weight of 24

kDa. This idea is supported by the fact that the 40-70% of total eluted proteins consisted of GroEL and GroES. Based on this theory, CAB with molecular weight of 30 kDa was a compatible substrate for the refolding study with our APCP containing mainly GroEL and ES. Furthermore, the CAB refolding efficiency with ACP was high, which may be due to ACP mimicking GroEL/ES system.

In this experiment, we used lon⁻ *E. coli* mutants to eliminate *E. coli* proteases. The ATP dependent ClpP protease of *E. coli* could be co-purified with chaperones from β-CN affinity column since this protease binds strongly to α-CN and degrades it in the presence of ATP (23). ClpP protease inhibitors, PMSF or DFP (diisopropyl fluorophosphate), were used to prevent ClpP proteolytic function since ClpP is a serine protease (35, 37). α-Casein also was added to the refolding mixture to trap ClpP protease (23). High salt (1 M NaCl) was used also to dissociate the interaction between ClpP and Clp proteins, since ClpP functions as a protease only by combination with Clp protein (21). The addition of protease inhibitors revealed that PMSF was the most effective protease inhibitor by increasing CAB refolding by 4-20%. Although DFP was as effective as PMSF, it was not used in each assay due to its strong toxicity (data not shown). α-Casein was not effective at increasing the refolding yield of CAB (H) or (C) after incubation with APCP and ATP.

In the refolding experiment, chemical was more efficient than thermal for CAB denaturation and CAB (H) was refolded to a higher yield (maximum of 98%) than CAB (C). It is possible that residual guanidine-HCl interfered in the refolding reaction. ACP was more efficient than APCP for refolded CAB, with a maximum of 116% activity. Commercial chaperones including DnaK, DnaJ, GrpE, GroEL, and GroES (Sigma) were added to a refolding assay to compare their refolding efficiency with ACP or APCP. Commercial chaperones added in combination did not show refolding activity for reasons that are not clear.

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# CHAPTER IV

# CONCLUSIONS

## HYPOTHESIS

Chaperones recognize unfolded polypeptide chains, the molten globule conformation, or hydrophobic sequences. The caseins are amphiphilic proteins with characteristics similar to the molten globule folding intermediate. Therefore, the caseins can be used to affinity purify the *E. coli* molecular chaperones.

This hypothesis was examined by completing a series of objectives to demonstrate affinity purification of molecular chaperones using immobilized caseins. Each component is associated with molecular chaperones purification efficiency.

The rationale for each objective is presented after the objective. Further details of the observations, result, and conclusions are presented in subsequent sections with the title for each objective. Finally, a conclusion relating the objectives to the hypothesis is presented.

## OBJECTIVES

1. To develop bioselective adsorption matrices for the affinity purification of molecular chaperones from *E. coli*. Both α-casein and β-casein will be immobilized to solid supports.

Caseins possess characteristics similar to the molten globule folding intermediate, which could be recognized by molecular chaperones. The milk proteins α-casein and β-casein were individually immobilized on to succinamidopropyl controlled-pore glass via carbodiimide. Covalent immobilization resulted in 45 mg protein/g beads for α-CN (α-casein) and 30 mg protein/g beads for β-CN (β-casein). These columns were used to prove the interaction between molecular chaperones and caseins in chapters II and III.

2. To compare the induction of *E. coli* chaperones with three treatments including heat stress, ethanol stress, and salt stress. Mild and severely stressful conditions will be applied for overexpression of molecular chaperones.

Two *E. coli* strains, NM522 and lon⁻ mutant BL21 (DE3) were used as the source of molecular chaperones. Two *E. coli* strains were grown to late log phase at 30°C for 17 h or concentrations of chaperones were increased with heat shock, ethanol stress, and salt stress. *E. coli* cells were induced by heat exposure at 39 °C for mild stress or at 42 °C for severe stress. The other stresses for chaperones expression were addition of ethanol (3% v/v), or salt (2% w/v) to the growth medium. The aim of this objective was to examine induction efficiency of molecular chaperones by various stresses, type of *E. coli* strain, and mild/severe

stress. The result from the objective was to provide the best source condition for molecular chaperones. The results of this objective are given in Chapters II, III, and Appendix A.

3. To determine the interactions and dissociation requirements between immobilized affinity matrices and purified molecular chaperones.

Previous reports provide the information related to interactions and dissociation between molecular chaperones and substrates. If the complex formed is mainly due to hydrophobic interactions, a decrease in temperature will lessen the strength of the association and an increase in ionic strength will strengthen the interaction. Complete dissociation of the complexes formed was determined by washing the column in 6 M urea to denature proteins and measuring proteins present in the eluant. This was done to test the efficiency of temperature or Mg-ATP to dissociate the complexes. The 6 M urea eluted chaperones were not used to refold proteins, rather they were used to show what proteins were bound between caseins and chaperones. Before elution of molecular chaperones, each casein column was washed with 1 M NaCl to remove nonspecifically bound proteins.

The purpose of this study was to characterize the interactions between chaperones and immobilized caseins. The data generated provided effects of Mg-

ATP, ionic strength, and temperature in the dissociation of the caseins-chaperone complexs. The results of this study are reported in Chapter II.

4. To compare source between two *E. coli* strains and column efficiency between affinity matrices (α-casein and β-casein) for the purification of molecular chaperones.

Two *E. coli* strains, NM522 and lon⁻ mutant BL21 (DE3) were induced by heat exposure at 39 °C or 42°C, addition of ethanol (3% v/v), or salt (2% w/v) to the growth medium to increase the concentrations of chaperones. The eluants from individual columns were analyzed by SDS-PAGE. The dried gel was measured by densitometry to measure the concentration of each molecular chaperone in samples. The quantity of each chaperone purified by each particular column using each *E. coli* cell was determined. The data generated in this objective provided the column efficiency of two matrices and source efficiency of two *E. coli* strains to purify the amount of individual chaperones in Chapter II.

5. To identify the purified molecular chaperones by Western analysis and N-terminal sequencing.

The members of two chaperone families (Hsp 70, Hsp 60) were identified by Western analysis since their antibodies are commercially available. The results of this study are shown in Chapter II and III. However, Hsp100 family

members were tried to confirm their identifications by N-terminal sequencing due to unavailability of their antibodies. The data generated in this objective provided the identification of molecular chaperones in eluants obtained from two matrices. Attempts were made to purify the Clp chaperones and data are shown in Appendix B.

6. To characterize purified molecular chaperones with respect to refolding activity of denatured CAB (carbonic anhydrase B) and ATPase activity.

Two types of assays were performed to characterize molecular chaperones obtained from objective 3. One assay, carbonic anhydrase B (CAB) was denatured by heating at 70 °C or incubation with 6 M guanidine-HCl. In a refolding experiment, denatured enzyme was incubated for several hours with affinity purified chaperones, 2mM ATP. CAB activity was monitored via pNPAc esterase assay spectrophotometrically over 60 sec at 400 nm in Chapter III. For the other assay, ATPase activity was assayed by liberation of inorganic phosphate from ATP after incubating chaperone molecules in10 mM MgCl, 1 mM ATP and 1 mM DTT for 30 min at 37 °C. The inorganic phosphate was measured at 660 nm in a spectrophotometer using phosphate standard (Biomol, Plymouth Meeting, PA). The result was used to prove functions of molecular chaperones, affinity purified from casein column in Appendix B.

Each objective defines specific aims to narrow the possible explanations of the hypothesis. The conclusions from each objective were used to guide the specific details for the next objective. As a whole, these objectives were used to test the validity of the hypothesis.

## CONCLUSIONS

The hypothesis was that caseins could be recognized by molecular chaperones due to caseins characteristics which are similar to the molten globule folding intermediate. Each objective provided independent evidence that supports specific components of this hypothesis. Taken together, the objectives collectively support and verify that the molecular chaperones were purified from both immobilized α- and β-casein columns. Purified molecular chaperones were effective at refolding denatured carbonic anhydrase B enzyme. Further work could be done to refold a different protein using affinity purified chaperones, or to purify Clp proteins using a casein column under optimized conditions.

# APPENDICES

# APPENDIX A

## AFFINITY PURIFICATION OF MOLECULAR CHAPERONES FROM SALT-INDUCED *E. COLI*

Covalent immobilization of β-CN resulted in 30 mg protein/g beads. Five milliliters of beads (2 g) were used for chaperone purification. Chaperone expression was induced by addition of media (25 °C) containing 2% NaCl (w/v) from the lon⁻ *E. coli* mutant, BL21. Approximately 10 mg of cell lysate protein was applied to an affinity column as the starting material.

Cold water (4 °C) was applied to dissociate the interactions between β-CN and chaperones since hydrophobic interactions are reduced at low temperatures. SDS-PAGE analysis of proteins eluted with cold water is shown in Fig. A. Lane L shows the protein profile of crude cell lysates treated at addition of 2% NaCl (T). Lane $H_2O$ shows proteins eluted with cold water from the β-CN affinity column after removing nonspecifically absorbed proteins with 1 M NaCl. Among the 5–8 bands, the major bands had the expected molecular weights of 4 different *E. coli* chaperones including DnaK (70 kDa), GroEL (57 kDa), DnaJ (41 kDa), and GroES (10 kDa) (Fig. A). Based on densitomity scanning of the SDS-PAGE analysis, the GroEL protein band in cold water eluate of 2% NaCl (T) constitutes about 50-60% of the total protein eluted, which is three times the

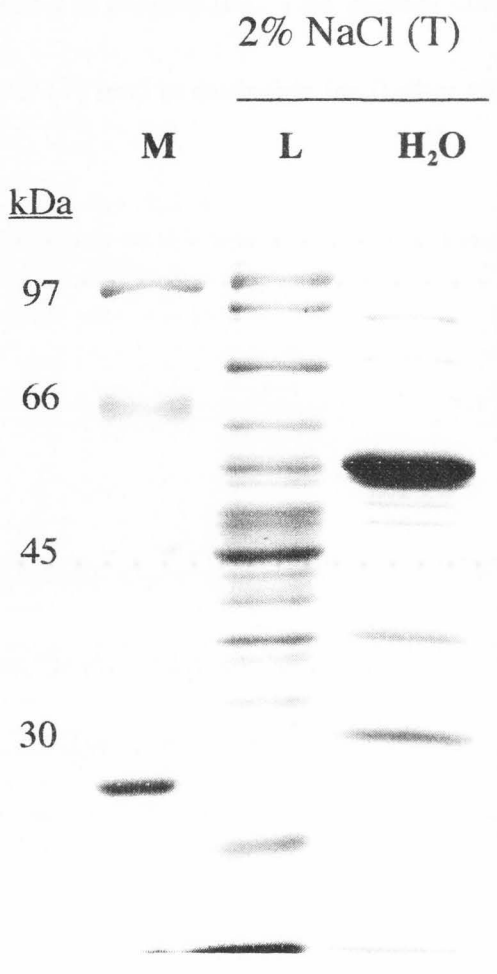


**FIG. A.** SDS-PAGE analysis of proteins purified from β-casein column. Approximately 20 μg of protein was applied for analysis. Salt was used for induction. Lane L, crude *E. coli* lysate; Lane H2O, chaperones eluted using cold water from cell treated with 2% NaCl (w/v). Lane M, protein molecular weight marker.

amount of the protein in cold water eluates of 39°C (T), 42°C (T) and 3% ethanol (T) (Fig. A, and 2 in chapter III). This unusual eluting pattern of GroEL in cold water eluate of 2% NaCl (T) lead to exclusion for further analysis in Chapter III.

# APPENDIX B

## AN ATTEMPT AT THE PURIFICATION OF CLP PROTEINS FROM *E. COLI*

### INTRODUCTION

The Clp (Caseino lytic protease or chaperone linked protease) proteins are found in both eukaryotes and prokaryotes (8). The Clp proteins are strongly induced in response to a variety of stressful conditions and their functions seem specific to conditions of stress (10, 12). The Clp proteins have roles in many cellular processes including protein reactivation, protein degradation, DNA replication, regulation of gene expression, thermotolerance, inheritance of prion-like factors, and protein translocation through membranes (11, 13). The Clp proteins (chaperone-linked protease or casein lytic protease) are important ATP-dependent and chaperone-linked proteases in *E coli* and named for their capacity to promote the proteolysis of casein (caseino-lytic protease) in vitro.

The ClpP protein consists of a tetradecamer with molecular weight of 21 kDa and composed of two stacked heptameric rings (17, 19). The *E coli* ClpA is a dimer of 84 kDa subunits and assembles to a hexamer in the presence of ATP (16). ClpB has a tetrameric ring structure of the 93 kDa subunit with a central cavity. ClpB has ATPase activity that is stimulated 5-10 fold by casein (13).

ClpP constitutes about 2% of the cellular protein (7). The amounts of ClpA, ClpX or ClpB in *E. coli* has been estimated at 1% of the soluble cellular proteins (2, 7, 21).

Purification of Clp proteins includes either centrifugation in a sucrose gradient, or sonication (2, 17) and followed by ammonium sulfate precipitation or salt precipitation (3, 7, 20) followed by ion-exchange chromatography (5, 6, 9) or size exclusion (7, 15, 21). The protein purity was increased by repeated ion-exchange (2, 5, 6) or hydrophobic interaction chromatography (1, 9).

Several affinity purification methods have been developed to purify ClpP using Ni-NTA agarose (17) or phosphocellulose column (6), ClpA using phophocellulose column and heparin agarose (5, 14), and ClpB using heparin agarose (4, 21).

In this study, I tried to purify Clp proteins by α-CN (α-casein) affinity chromatography as well as by Mono Q anion exchange chromatography and ATPase assay.

*Approach 1.*

Covalent immobilization of α-CN resulted in 45 mg protein/g beads. Five milliliters of beads (2 g) were used for chaperone purification. As the source of or Clp protein, $Lon^-$ mutant BL 21 strain was used, *E. coli* cells were induced for chaperone expression with heat exposure at 46 °C not 42 °C as described by Parsell et al., (10).

Major chaperone are induced in response to mild stress but Clp protein, in contrast, is strongly induced in response to severely stressful conditions (10). Approximately 10 mg of cell lysate was applied to an affinity column as the starting material. Mg-ATP (1 mM) or α-CN (1 μM) was applied to dissociate the interactions between α-CN and Clp proteins since Clp proteins are well known to bind with high affinity to α-CN and degrade it with ATP hydrolysis (9, 18).

SDS-PAGE analysis of proteins eluted with 1 μM α-CN or 1 mM Mg-ATP is shown in Fig. B-1. Lane L shows the protein profile of crude cell lysates treated at 46°C. Lanes ATP and α-CN show proteins eluted with α-CN or Mg-ATP from the α-CN affinity column after removing nonspecifically absorbed proteins with 1 M NaCl. There is an enrichment in proteins between 97 kDa and 30 kDa (Fig. B-1, Lane L). The major band had the expected molecular weight of GroEL (57 kDa). The ATP eluate showed a protein band with expected molecular weights of ClpA (84 kDa) or ClpB (93 kDa). Both ATP and α-CN eluates contained the enriched GroEL protein band with an expected molecular weight of 57 kDa. The darkest band from α-CN eluate was not ClpP (23 kDa) but α-CN (24 kDa) added as the eluting agent.

*Approach 2.*

ATP eluate (Fig. B-1, Lane ATP) was applied to Mono Q anion exchange column

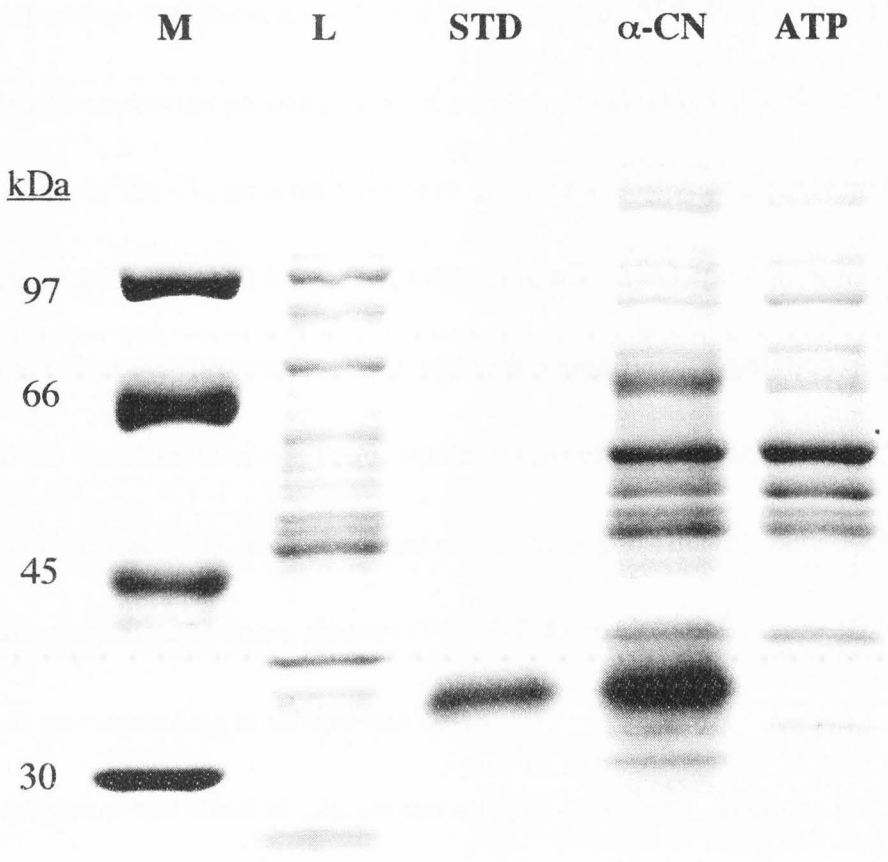


**Fig. B-1.** SDS-PAGE analysis of proteins eluted from α-casein column. Approximately 20 μg of protein was applied for analysis. Heat shock at 46 °C was used for induction of chaperones from BL21 *E. coli* strain. Lane L, crude *E. coli* lysate; Lane STD, 2 μg of commercially purchased α-CN; Lane ATP, proteins eluted using 1 mM ATP; Lane α-CN, proteins eluted using 1 μM α-CN; Lane M, protein molecular weight marker.

for charge separation and fractions collected for analysis by SDS-PAGE (Fig. B-2). Fractions 8 to 11 shows the protein profile of proteins eluted at 0.3-0.4 M NaCl. Previously, most of the Clp proteins have been purified with salt gradient from 0.3 M (ClpA, ClpX, and ClpP) to 0.4 M NaCl (ClpB) using a Mono Q column (5, 6, 9).

Anion exchange chromatography resulted in a significant separation of *E. coli* proteins and the enrichment of one band, which has the expected location of either ClpA or ClpB (Fig. B-2, Lane 11). Fraction 8 had a prominent protein with same location of GroEL in commercial chaperones mixture (Fig. B-2, Lane Cpns). Fraction 10 had protein bands corresponding to GroEL and DnaK.

To determine the effect of Clp protein on ATP hydrolysis, fractions from Mono Q column were assayed for ATPase activity using protein phosphatase kit (Biomol, Plymouth Meeting, PA). ATPase activity was assayed by liberation of inorganic phosphate from ATP after incubating chaperone molecules in10 mM MgCl, 1 mM ATP and 1 mM DTT for 30 min at 37 °C. The inorganic phosphate was measured at 660 nm in a spectrophotometer using phosphate standard (Biomol, Plymouth Meeting, PA). Approximately 5 μg of protein from each fraction was used for ATPase assay (Fig. B-3). As expected, fraction 11 showed the highest ATPase activity among samples. Therefore, one dark band from fraction 11 (designated by arrow) was analyzed by N-terminal sequencing for identification.

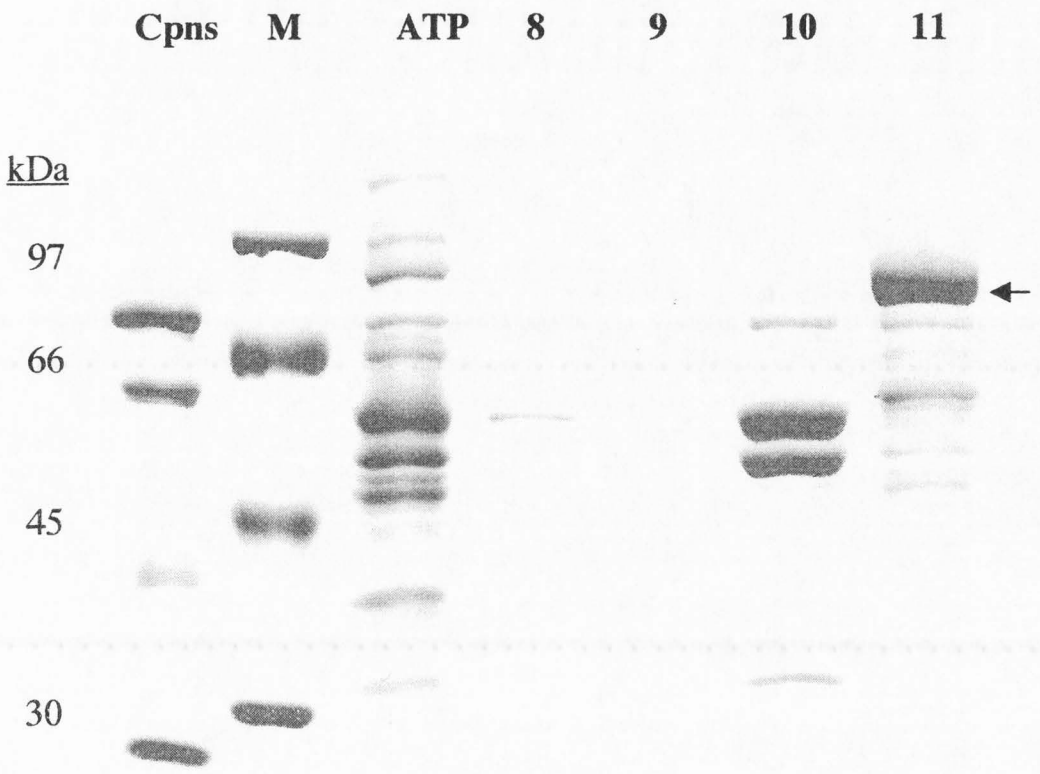


**Fig. B-2.** SDS-PAGE analysis of Mono Q HPLC fractions. Arrow designate the protein band analysed by N-terminal sequencing. Approximately 20 μg of protein was applied for analysis. Lane Cpns, total 10 μg of commercially purchased chaperones including DnaK, GroEL, DnaJ, GrpE, and GroES; Lane ATP, proteins eluted using 1 mM ATP; Lanes 8-11, fractions collected with 0.3-0.4 M NaCl in 50 mM Tris-Cl, pH 7.5 buffer from Mono Q column; Lane M, protein molecular weight marker.

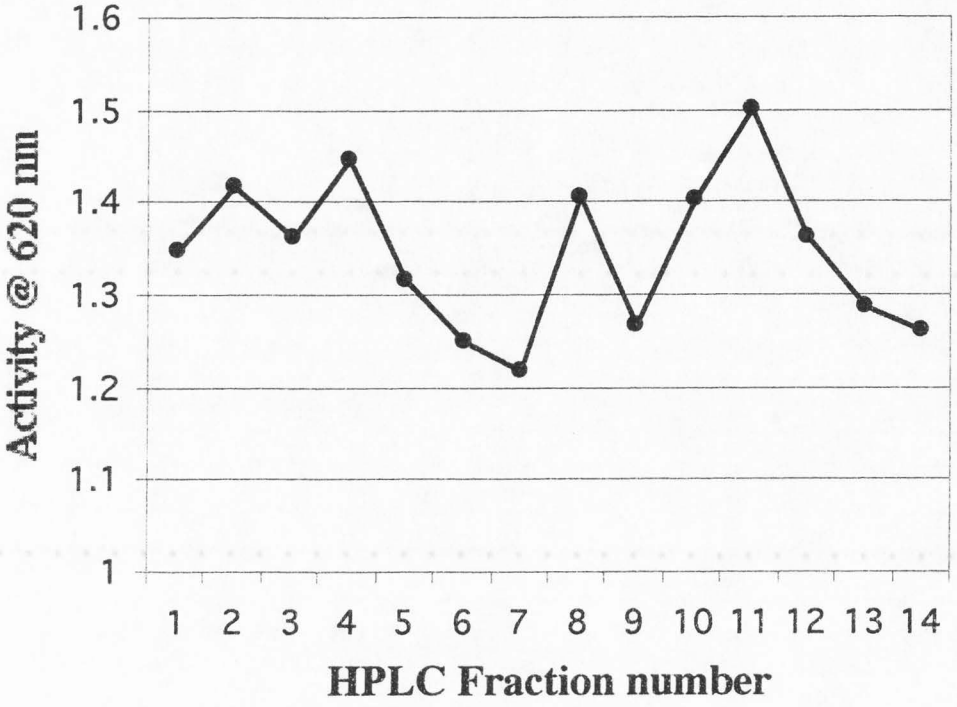


**Fig. B-3.** ATPase activity of fractions collected from Mono Q HPLC. Approximately 5 ug of protein from each fraction was incubated with 2 mM ATP for 10 min at 37 °C. Protein phosphatase (Biomol, Plymouth, PA) was added to quench the activity and the amount of released inorganic phosphates ions was measured spectrophotometrically at 620 nm.

The result revealed that the protein band within 70 kDa to 97 kDa (Fig. B-3, Lane 11) was identical with PNPase (polynucleotide phosphorylase).

Due to time limitations and the lack of detection equipment for Clp protein, further investigation was terminated. Immobilized α-CN developed from approach 1 was used to fractionate chaperones from *E. coli* lysate. Mono Q anion exchange chromatography and ATPase assay from approaches 2 contributed mainly to isolate PNPase (polynucleotide phosphorylase) from *E. coli* lysate.

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   Third place–IFTSA National Product development Competition.

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1. Walsh, M. K. and Nam, S. H. (1999) Affinity purification of lactoferrin transferrin, prosaposin, and PP3 from milk and whey. (Provisional patent)

2. Nam, S. H. and Walsh, M. K. (2003) Characterization of interaction between *E. coli* molecular chaperones and immobilized caseins. In preparation of maunscipt for Biosci. Biotech. Biochem.

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6. Nam S.H. and M.K. Walsh. June 1998. Affinity purification of prosaposin from whey. Institute of Food technologists Annual Meeting, Atlanta, GA.

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