Improving the recovery of ‘difficult to release’ periplasmically-expressed products from recombinant *E. coli*

By

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Thesis Abstract

The periplasm of *E. coli* has been recognized a suitable location for the production of large quantities of many industrially important soluble recombinant proteins, and offers some important advantages over both intracellular and extracellular production. The oxidative environment of the periplasm promotes correct disulphide bonding and protein folding; there is reduced risk of proteolytic attack in the periplasm; and the periplasm accounts for <5% of total cell protein, so that selective release reduces subsequent purification demands. Despite these merits, periplasmic expression systems have not yet fulfilled their true potential, largely due to the lack of reliable general methods for efficient selective release of periplasmically expressed proteins at large-scale. The classical osmotic shock procedure, the only reliable method for releasing proteins from the periplasmic space of *E. coli*, is expensive and time-consuming, and thus it is not feasible at large scale. The main objective of this study has been to develop a gentle chemical permeabilisation method for selective release of periplasmically-expressed proteins from recombinant *E. coli*. In the first experiments, the titre, location and form of anti-lysozyme Fab D1.3 were determined during fed-batch cultivation of *E. coli*. It was shown that the Fab produced as both soluble and insoluble forms and released into the culture medium over the course of fed-batch fermentation. Purification of Fab D1.3 was then performed using various chromatographic methods, and the most effective target Fab purification was achieved by using sequential cation exchange – Protein G affinity chromatography route with an overall yield of 83%. Pure model proteins typically secreted to the periplasm (i.e. beta-lactamase, alpha amylase and Fab D1.3 fragment) were subsequently exposed to various chemicals, and alterations in the secondary structure of the proteins in the presence of various chemicals were investigated by high throughput circular dichroism (ht-CD) system. Chemicals such as 0.1% Triton X-100, 0.05% benzalkonium chloride (BAC), 0.1% cetyltrimethylammonium bromide (CTAB), and 2 M urea remarkably changed the secondary structure of beta-lactamase and

alpha-amylase. The secondary structure of Fab D1.3 was more vulnerable to the tested chemicals. The biological activity of the target proteins in the presence of various chemicals was also measured and it was revealed that changes in the secondary structure of proteins do not necessarily cause reduction in the biological activity and vice versa. Concentrations of chemicals which did not reduce the biological activity of the proteins were eventually examined in subsequent periplasmic release experiments using recombinant *E. coli* strains producing the same target proteins, and the performance of various chemical permeabilisers were evaluated by comparing to classical osmotic shock and mechanical cell disruption. It was demonstrated that low concentrations of chemicals such as sodium deoxycholate (DOC) and/or chelating agents, isoamyl alcohol released the periplasmic proteins as efficient as or more efficient (up to 168%) than osmotic shock treatments. It was also proved that chemicals could increase the periplasmic release efficiency when they used in combinations. For instance, 1 M EDTA in combination with detergents could increase the periplasmic release of beta-lactamase and Fab D1.3 up to 80% and 130%, respectively. Such synergetic effect for release of alpha-amylase and Fab D1.3 was also observed when 1% solvents (hexane, xylene, benzene, toluene, and isoamyl alcohol) were combined with detergents such as 0.025% DOC, 0.01% CTAB and 0.1% Triton X-100.

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List of abbreviations

AC: Affinity chromatography
AEX: Anion exchange
AUC: analytical ultracentrifugation
BAC: Benzalkonium chloride
Bicinchoninic acid: BCA
BSA: Albumin from bovine serum
CD: Circular dichroism
CEX: Cation exchange
CFU: colony forming units
C_H: Constant heavy chain
C_L: Constant light chain Disulphide bond-forming: Dsb
CTAB: Cetyltrimethylammonium bromide
DCW: Dry cell weight
DDH_2O: Double distilled water
DEA: Diethanolamine
DOC: Sodium deoxycholate
EDTA: Ethylenediaminetetraacetic acid
ELISA: Enzyme-linked immunosorbent assay
Fab: Fragment antigen binding
Fc: Fragment crystallizable
Fv: Fragment variable
GM-CSF: Granulocyte macrophage colony stimulating factor
GndCl: Guanidinium chloride
GST: Glutathione *S*-transferase
HEWL: Hen egg white lysozyme
HT: High tension
IB: inclusion body
IEC: Ion exchange chromatography
Ig: Immunoglobulin

LB: Luria Bertani

LPS: lipopolysaccarides

MBP: Maltose-binding protein

MES: 2-(N-Morpholino)ethanesulfonic acid

mRNA: Messenger ribonucleic acid

NTA: Nitriolotriacetic acid

OD: Optical density

OM: Outer membrane

Omp: outer membrane proteins

OS: Osmotic shock solution

PBS: Phosphate buffered saline

PEG: Polyethylene glycol

PF: Purification factoy

PPIase: peptidyl-prolyl isomerase

scFv: Single-chain variable fragment

SDS: Sodium dodecyl sulphate

SDS-PAGE: SDS-poly-acryl amide gel electrophoresis

SHMP: Sodium hexametaphosphate

SRP: signal recognition particle

Tat: *t*win *a*rginine *t*ranslocation

TEMED: Tetramethylethylenediamine

TF: trigger factor

TNF: Tumour necrosis factor

Tris: Hydroxymethyl)aminomethane

V_H: Variable heavy chain

V_L: Variable light chain

V_Hs: Single domain antigen binding fragment

YF: Yield factor

List of symbols

A_λ	Optical density or absorbance
I_0	Intensity of light entering the sample
ε	Molar extinction coefficient $(moles\text{-}cm)^{-1}$
C	Concentration $(moles\ L^{-1})$
l	Length (cm)

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1 Introduction

1.1 Manufacture of recombinant proteins in *E. coli*

Escherichia coli is a very well studied bacterium, and since the advent of recombinant DNA technology has been the host of choice for the manufacture of many non-glycosylated proteins (Blattner *et al.*, 1997; Choi and Lee, 2004; Schumann and Ferreira, 2004). There are three locations for the manufacture of proteins in *E. coli*: the cytoplasm, moving into the culture media (excretion), and moving into the periplasm (secretion).

Proteins are usually over-expressed for high-level production which is a prerequisite for subsequent purification. A frequent consequence of the over-expression of eukaryotic proteins in the cytoplasm of *E. coli* is the accumulation within the cell of insoluble bodies known as inclusion bodies (IBs; Ventura and Villaverde, 2006). IBs can contain the target protein in up to 90% pure form, and insolubility appears to result from the formation of intracellular protein aggregates of misfolded product (Carrió and Villaverde, 2002; Heebøll-Nielsen *et al.*, 2003). Complete denaturation is essential for the efficient solubilisation of the recombinant product and specific renaturation conditions are required for significant yield recovery of the soluble protein (Radolph and Lilie, 1996; Vallejo and Rinas, 2004).

The physical properties of IBs (strong, large, dense and highly refractile particles with defined size and optical properties) are exploited to good effect in industrial recovery and monitoring processes (Fischer *et al.*, 1993; Georgiou and Valax, 1999; Jin *et al.*, 1994; Singh and Panda, 2005; Taylor *et al.*, 1986). The conventional route for purifying proteins produced in IB form follows a lengthy and highly conserved sequence of operations involving mechanical cell disruption, separation of the insoluble IBs using centrifugation or diafiltration, washing, solubilisation using a highly concentrated chaotrope such as 8 M urea or guanidine hydrochloride, and eventually several chromatographic purification stages followed by dilution refolding of the target protein (Singh and Panda, 2005). Not surprisingly, therefore,

manufacturing a protein by an IB route is very costly and tends to be reserved for high-value human biotherapeutic agents.

In molecular terms secretion is simply defined as the movement of a protein across a cell membrane or envelope either in the extracellular culture medium and/or within the cell which in the case of *E. coli* is the periplasm (or periplasmic space). Promoters and secretion signals from a few outer membrane proteins (e.g. OmpA and OmpF) and naturally occurring periplasmic proteins (e.g. alkaline phosphates and beta-lactamase) have been used to direct extracellular and periplasmic secretion respectively (Table 1.1). Targeting protein to the extracellular culture medium (excretion) can facilitate downstream processing. With ATP hydrolysis, excretion of proteins to the extracellular culture medium of *E. coli* can be accomplished by the type I secretion mechanism (HlyA/HlyD/TolC pathway), or by the type II mechanism mediated by some secreton components named as main terminal branch (MTB) components (Mergulhao *et al*., 2005; Pugsley *et al*., 1997; Sandkvist, 2001). Proteins can also reach the culture medium by nonspecific periplasmic leakage during cell division and accumulation of recombinant protein in the periplasm which builds up an osmotic pressure (Mergulhao *et al*., 2005). Despite its many advantages (the most obvious being minimum product contamination by host proteins as well as no requirement for cell disruption; Mergulhao *et al*., 2005) development of *E. coli* as a useful host for the excretion of commercially important proteins to the culture broth suffers major drawbacks. The most serious obstacle is the structure of the cell envelope of *E. coli* (Fig. 1.1), which in the majority of cases leads to the proteins ending up in the periplasm rather than the culture medium. Furthermore, when the product is excreted into the culture medium, it becomes highly diluted, which demands not only the use of high volume capture or concentration steps, but it can also result in considerable physical damage as a direct consequence of the shear forces generated by some microbial fermentation processes (Humphreys, 2007).

The periplasm, on the other hand, seems a much better location for the production of large quantities of soluble recombinant proteins (Joly *et al.*, 1998; Laird *et al.*, 2005; Carter *et al.*, 1992; Chen *et al.*, 2004), and proffers advantageous over the cytoplasmic route. The oxidizing environment of the periplasm in cooperation with

Table 1.1 Heterologous protein secretion in *E. coli*. [a]Is a theoretical value at specific cell density of OD_{600}=60 and assumes a linear correlation between biomass and yield (in reality in very few cases production has been measured at such high cell density); [b]EE-IGF-1 is a fusion protein; IGF-1 (insulin-like growth factor) was fused to two copies of the E-domain of *S. aureus* protein A; the promoter & secretion signal are also from protein A; [c]Precise location of bPTI is unclear because it is not released from the cells by cold osmotic shock procedures (Table taken from Moir and Mao, 1990).

Protein	Secretion signal	Promoter	Projected yield[a] (mg/L)	Location
EE-IGF-1[b]	proA	proA	190	broth
Chicken Ovalbumin	ovab	Lac	90	periplasm
Epidermal Growth Factor (EGF)	phoA	phoA	75	periplasm
Staphylococcus aureus nuclease	ompA	Lpp	30	periplasm
β–endorphin	ompF	ompF	28	broth
β–endorphin	ompF	trp	24	broth
Bovine pancreatic trypsin inhibitor (bPTI)	phoA	phoA	7.8	periplasm ?[c]
Human Growth Hormone (hGH)	ompA	Lpp/Lac	0.9	periplasm
Proinsulin	bla	bla	0.006	periplasm

periplasmic proteins assists the formation and isomerisation of disulphide bonds, resulting in a firmly folded structure resistant to proteases (Berkmen e*t al.*, 2007; Derman and Beckwith, 1991). Chaperon properties of certain periplasmic proteins can also hamper the aggregation of target products (Betton, 2007; Richarme and Caldas, 1997). Furthermore, the outer membrane of gram negative bacteria is penetrable to small (≤ 600 Da) solutes (Nakae and Nikaido, 1975), and as a result the periplasm can be manipulated by being exposed to extracellular chemical agents, which assist the folding and modification of periplasmically-expressed proteins (Barth *et al.*, 2000; Sandee *et al.*, 2005; Wunderlich and Glockshuber, 1993). Additionally, the periplasmic space of *E. coli* contains only 4-8% of the total cell protein (Park and

Lee, 1998) whereas problematic cytoplasmic components (particularly nucleic acids and proteases), which pose additional purification challenges during product recovery, are not present.

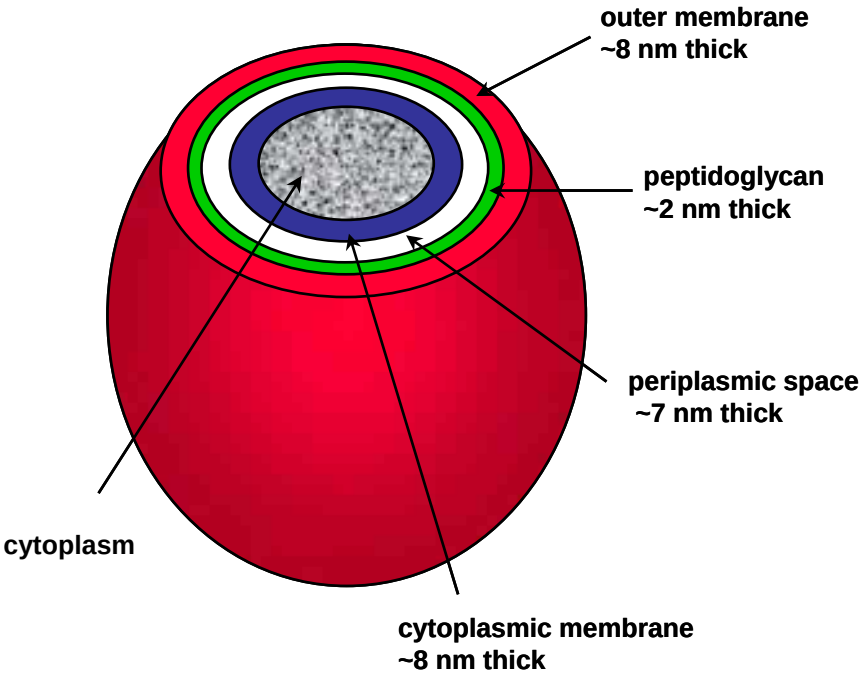

Fig. 1.1 Schematic illustration of the envelope structure of *E. coli* and other gram-negative bacteria.

1.2 Periplasm

1.2.1 History

Periplasm is a separate compartment within gram-negative bacterial cells, with its singular components and characteristics. The exceptional disposition of the periplasm has been disclosed through many research works. The existence of distinctive periplasmic proteins was proved by Harold Ne and Leon Heppel in 1964 (Neu and Heppel, 1964). These scientists demonstrated the release of ribonuclease from *E. coli* cells by converting them into spheroplasts by the EDTA-lysozyme treatment in sucrose. Prior to these studies it was not manifest that how *E. coli* could endure the presence of destructive enzymes such as ribonuclease and phosphates

which could raze vital components of the living cells, the existence of which was obvious by releasing upon cell breakage. Neu and Heppel discovery showed that they were kept in the periplasmic space, away from the cytosol, in gram-negative bacteria.

The name ‘outer membrane’ was used for the first time in the electron microscopy study of a periodontal pathogen *Veillonella* by Howard Bladen and Stephan Mergenhagen in 1964 (Bladen and Mergenhagen, 1964), the year in which the release of periplasmic ribonuclease was ascertained by Neu and Heppel.

Donnan potential across the outer membrane was found out in 1977 (Stock *et al*., 1977), illustrating that the periplasm and its surrounding environment are dissimilar in terms of their composition of small molecules. The inside-negative potential results in the accumulation of small cations in the periplasm. The presence of polyanionic oligosaccharides in periplasm, responsible for the generation of Donnan potential, was demonstrated by Howard Schulman and Eugene Kennedy in 1979 (Schulman and Kennedy, 1979). The two barriers of the periplasm are not fully detached; for instance, “adhesion sites” between the two barriers were discovered in 1968 (Bayer, 1968). The periplasm makes cells robust and competitive, and has numerous similarities with the endoplasmic reticulum of eukaryotic cells, with regard to protein transfer and folding. The periplasm has been attracting attentions for production of recombinant proteins containing disulphide bonds.

1.2.2 Protein secretion to the periplasm of *E .coli*

It has been approximated that 450 proteins are exported across the cytoplasmic membrane in *E. coli* (Palmer and Berks, 2007). Two distinct pathways exist for the secretion of protein into the periplasm of *E.* coli. The majority of the secreted proteins are transported in an unfolded form via the general secretory (Sec) pathway (Fig. 1.2). A fundamentally different pathway for periplasmic protein localization in *E. coli* is

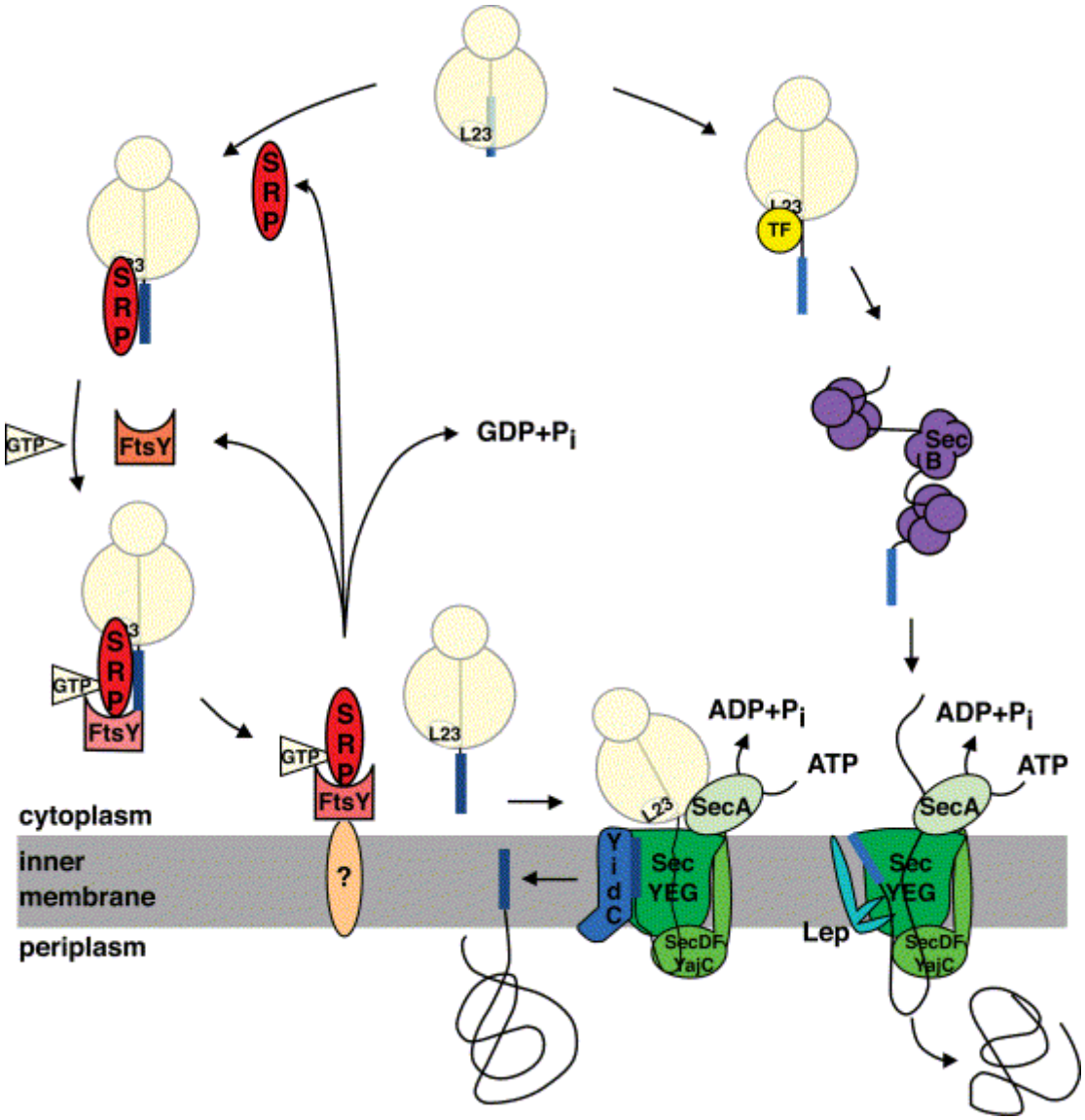

Fig. 1.2 The SRP and Sec targeting pathways in *E. coli*. The SRP binds to a specific highly hydrophobic signal sequence in nascent proteins at L_{23}. FtsY binds to the ribosome-SRP complex and supports targeting to the inner membrane through its affinity for lipids and, probably, a proteinaceous membrane factor. In a GTP-dependent reaction, the nascent protein is transferred to the Sec-translocon. TF, a cytosolic protein, interacts with less hydrophobic signal sequence in nascent polypeptide at L_{23} and may affect the interaction of the SRP. Sec B binds to the secretory proteins with a mild hydrophobic signal peptide and guides this to the Sec-translocon which is identical to the translocon via the SRP-pathway. The model is not drawn to scale (Figure taken from Luirink and Sinning, 2004).

the *t*win *a*rginine *t*ranslocation (Tat) pathway, in which Arg-Arg motif found near the N- terminus of the leader peptide of folded proteins are engaged in the export of from the cytoplasm.

In the Sec pathway, co- and posttranslational targeting systems are the two pathways, joining at the SecYEG translocon located in the cytoplasmic (inner) membrane, in *E. coli*, through which targeting of secretory and membrane proteins takes place (Fig. 1.2).

Although many secretory proteins in *E. coli* use the posttranslational pathway, some of them such as inner membrane proteins (IMPs) are inserted/translocated through the cotranslational system. The cotranslational pathway is mediated by the signal recognition particle (SRP) system, which is responsible for the targeting of ribosomes translating SRP substrates to the SecYEG translocon. Cotranslational (SRP) system in *E. coli* involves two essential proteins, namely Ffh and FtsY, and 4.5S RNA. Proteins chosen for SRP system, which include inner membrane protein of *E. coli*, have a highly hydrophobic N-terminal (signal peptide). Various domains of Ffh are engaged with hydrophobic signal peptide in the nascent polypeptide, with the 4.5S RNA, with the ribosome and with FtsY. Ffh binds to the signal peptide of the polypeptides as they emerge from the ribosome, but not if they have been fully released from the ribosome. The cotranslational targeting is started by the interaction of Ffh with L_{23} ribosomal protein, near the nascent chain exit site, and with hydrophobic signal peptide as it comes out from ribosome; interaction which requires 4.5S RNA and results in a ribosome-SRP complex. FtsY binds to this complex, being GTP-dependant, and support targeting to the inner membrane through its affinity for inner membrane. GTPase activity of both Ffh and FtsY leads to the GTP hydrolysis, which is a fundamental step for dissociation of SRP from the ribosome translating nascent chain and for transferring the growing protein to the Sec-translocon (SecYEG complex).

However, most secretory proteins in *E. coli* are first synthesised in the cytoplasm and then targeted to the translocon via posttranslational targeting, in which specific chaperones are involved. TF (trigger factor), which is a cytosolic protein, is engaged

in posttranslational pathway. Both SRP and TF recognize and attach to the hydrophobic signal sequences in the growing polypeptide at ribosomal L_{23} protein, but TF interacts with less hydrophobic ones. Upon release of the TF-polypeptide-complex from the ribosome, SecB chaperone interact with the polypeptide, leading to the dissociation of TF. SecB also hinders protein premature folding and aggregation. For appropriate targeting to the translocon, the Sec B-preprotein complex must interact with SecA which is the membrane peripheral ATPase subunit of the translocon; the interaction after which the SecB is released from the preprotein. ATPase activity of SecA mediates the translocation step in the context of the SecYEG translocon.

Outer membrane proteins (OMPs). OMPs are targeted to the cytoplasmic membrane via the posttranslational (Sec B/Sec A) pathway after being biosynthesised, and next transported in an unfolded form into the periplasm via the Sec YEG translocon. The signal sequences of the OMPs are removed by peptidase. The unfolded periplasmically-located OMPs are kept soluble by the action of periplasmic chaperones, having been demonstrated to function without requiring ATP in their catalytic cycles – as there is no ATP in the periplasm. Overproduction of OMPs, accumulation of unfolded OMPs or misfolded OMPs (for example, as a consequence of stress such as heat) in the periplasm activates the stress σ-factor, σ^E (RpoE), in the cytoplasm, which subsequently results in production of periplasmic chaperones (such as Skp, SurA, FkpA, and Deg) and proteases (such as DegP). After accurate folding in the periplasm, OMPs are targeted to or assembled into the outer membrane by a process, the mechanism of which has not been fully known (Kleinschmidt, 2007; Mecsas *et al*., 1993).

A subset of secreted proteins in *E. coli* uses the Tat pathway for translocation to the periplasm. The Tat protein export pathway involves Tat A, B, and C components (Fig. 1.3). It has been demonstrated that 26 *E. coli* proteins have Tat signal peptides, the most distinctive characteristic of which is having consecutive (or twin) arginine residues. The Tat pathway is responsible for translocation of proteins that are incompatible with the Sec pathway such as those requiring the association of subunits or integration of cofactors before export (Delisa *et al*, 2003; 1996; Palmer

and Berks, 2007). For these Tat substrates, there is a complex chain of events that must happen prior to transport of the proteins to the periplasm (Palmer and Berks, 2007). For example, proteins must bind their cofactors in the cytoplasm prior to transport, and this binding requires the proteins to fold (this is why these class of

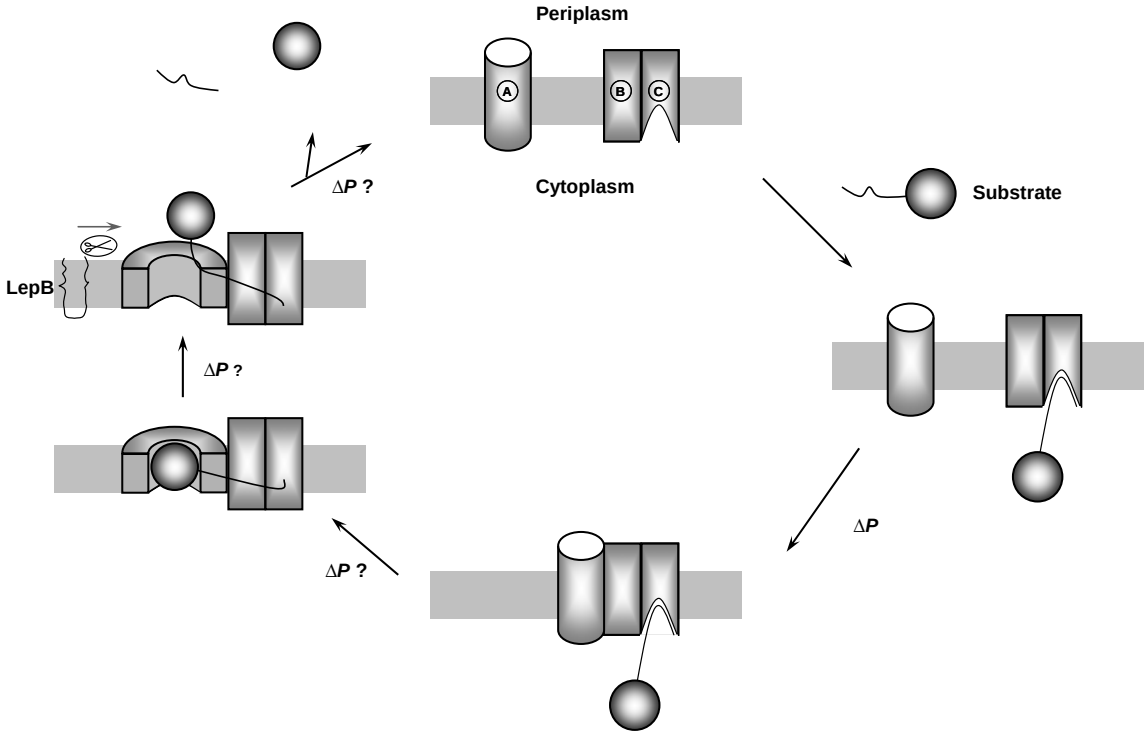

Fig. 1.3 The Tat translocation pathway. In the resting state, TatA and TatBC form separate, oligomeric complexes (top). The twin-arginine motif of the signal peptide of a substrate binds to a site in TatC (right). The TatBC-substrate complex now interacts with TatA in an energy-dependent step (bottom). The substrate is transported through a channel made up of TatA. The energetic requirements for this are unknown (left). During the transport cycle the signal peptide is cleaved by signal peptidase LepB, and the exported protein is released at the periplasmic face of the membrane. The TatA and TatBC components dissociate and the system returns to the initial state (top). (Figure redrawn from Berks *et al*., 2005).

proteins cannot use the Sec pathway for transport). In addition, many of the Tat substrates are exported as heterodimers or heterotrimers, while the twin-arginine-containing signal exists on only one of the subunits. In this case, non-signal bearing

subunit(s) should attach to the signal-bearing subunit prior to transportation via Tat pathway. The cell wall amidases are another major class of proteins exported by the *E. coli* Tat system (Berks, 1996; Palmer and Berks, 2007). These classes of proteins do not contain cofactors, and it is not clear why these proteins must require the Tat pathway for their transport to the periplam.

1.2.3 Disulphide bond formation and modification in the periplasm

Three proteins including alpha-amylase, beta lactamase, and Fab D1.3 – all expressed in the periplasm of *E. coli* – were studied in this thesis.

Alpha-amylase is an enzyme that hydrolyses alpha-bonds of large alpha-linked polysaccharides such as starch and glycogen, yielding glucose and maltose. Alpha-amylase from *Aspergillus oryzae* (EC 3.2.1.1; α-1,4-glucanohydrolase) is a monomeric enzyme containing four disulphide bonds – linking cysteine residues 30–38, 150–164, 240–283, and 439–474 – and one free sulfhydryl group in its tertiary structure (Kawata *et al*., 1998; Matsuura *et al*, 1984).

Beta-lactamases are bacterial enzymes (EC 3.5.2.6) that catalyze the hydrolysis of the beta-lactam ring of some penicillins and cephalosporins, producing penicilloic acid and rendering the antibiotic ineffective. Plasmid pBR322-encoded beta-lactamase contains a single disulphide bond linking cysteine residues 52–98 (Pollitt and Zalkin, 1983).

Fab fragments typically contain two intrachain disulphide bonds in the variable domains (V_H and V_L) of the F_V fragment and two disulphide bonds in constant domains (C_L and C_H1) plus one (or more) disulphide bridge which connects the heavy and light chains (Kikuchi *et al*., 1986; Venturi *et al*., 2002). According to communications with Merck Sharp and Dohme Ltd., the supplier of the *E. coli* strain producing anti-hen egg white lysozyme Fab D1.3 in the periplasm, there is no interchain disulphide bond between heavy and light chains of the Fab produced in this strain.

Disulphide bonds generally contribute to the three-dimensional structure of a protein, and are essential for the folding, stability, and full activity of many secreted proteins (Nakamoto and Bardwell, 2004). Disulphide bonds diminish the conformational entropy leading to increase protein stability. For instance, alkaline phosphatise, a periplasmic protein, contains two disulphide bonds necessary for its correct folding, in the absence of which the protein is almost degraded *in vivo* (Bardwell *et al*. 1991). The formation of disulphide bonds is a crucial posttranslational modification directed by an oxidation process in which the thiol (SH) groups of two cysteines are connected by covalent linkage. In some proteins, disulphide bonds can be obliterated through a reductive process resulting in two free cysteines, or can be rearranged (i.e., the array of disulphide bonds is changed) via a process called disulphide bond isomerisation (Berkmen *et al*., 2007).

Historically, Anfinsen and colleagues studied ribonuclease A (RNase A), containing four disulphide bonds all of which required for its full activity, and demonstrated that denatured and completely reduced RNase A could refold spontaneously to its active oxidized form in the presence of molecular oxygen (Anfinsen and Haber, 1961; Anfinsen *et al*., 1961; White, 1960). Goldberger and colleagues illustrated that the rate of disulphide bonds formation *in vivo* was extremely faster than that in vitro; the in vitro yield of corrected folded protein being low, with much of the protein containing the incorrect disulphide bonds (Berkmen *et al*., 2007; Goldberger *et al*., 1963). Hence, it was conjectured that there might be a biological system that corrected the erroneous disulphide bonds and led to the formation of native disulphide bonds. Protein disulphide isomerase (PDI), the eukaryotic endoplasmic reticulum enzyme, involved in this process was discovered in 1966 (De Lorenzo *et al*., 1966). For many years it was deemed that cells only needed the isomerase activity for disulphide bond formation, until divulging the periplasm disulphide bond-forming (Dsb) proteins in *E. coli* (Fig. 1.4) which were found to be integral for accurate disulphide bond formation of envelope proteins (Akiyama *et al*., 1992; Bardwell *et al*., 1991).

Today, it is evident that disparate *in vivo* modifications are catalyzed by the function of a group of enzymes named disulphide oxidoreductases and isomerases which are existed in all domains of life (Berkmen *et al*., 2007). Disulphide bond formation and

isomerisation take place in dedicated oxidizing compartments in organisms; in gram negative bacteria being the periplasm. However, proteins with disulphide bonds are scarcely found in the cytoplasm of gram negative bacteria; for instance, the cytoplasmic enzyme ribonucleotide reductase. Disulphide bonds in such proteins are evanescent, being formed and reduced in the pathway of participating in electron transfer reactions (Berkmen *et al*., 2007; Prinz *et al*., 1997).

Proteins necessitating disulphide bonds for their stability and activity are thought to be transferred into the periplasm co- or posttranslationally, via the Sec YEG translocon as discussed earlier, with their cysteines in a reduced state. In the cotranslational pathway, export of protein instigates shortly after initiation of its biosynthesis, eschewing cytoplasmic folding. In the posttranslational pathway, the attachment of antifolding agents, such as SecB protein, to the polypeptide retains them in an unfolded state and makes them export-competent. The construction of disulphide bonds between cysteines in proteins is catalyzed by periplasmic DsbA, a monomeric soluble protein analogous to thioredoxin. DsbA itself is tranlocated into the periplasm via cotranslational pathway (Schierle *et al*., 2003), while most periplasmic proteins are translocated posttranslationally. The cotranslational export of DsbA seems to be crucial inasmuch as the protein folds quickly in the cytoplasm (Huber *et al*., 2005; Schierle *et al*., 2003). Appearing in the periplasm with reduced cysteines in its active sites, DsbA is oxidized by the membrane protein DsbB in order to function as a donor of disulphide bonds. Reduced DsbB is oxidized by ubiquinone or menaquinone, respectively, under aerobic and anaerobic conditions. It has been approximated that at least 90 per cent of the periplasmic proteins contain disulphide bonds, being operated on by DsbA (Berkmen *et al*., 2007).

The gene dsbA is expressed constitutively in the periplasm at a basic rate of about 850 molecules per cell; the expression of which is augmented in response to cell envelope-folding defects resulting from stress (Akiyama *et al*., 1992; Berkmen *et al*., 2007). There is evidence that DsbA may indiscriminate in forming disulphide bonds in substrate. “Consecutive” model of disulphide bond formation can elucidate the reason for construction incorrect disulphide bonds. According to this model, DsbA reacts with the first cysteine of the polypeptide chain crossing into periplasm and link

it in a disulphide bond with the next one to emerge. Therefore, the product of interaction of DsbA with protein, in which four cysteines exist, would contain disulphide bonds between cysteines 1 and 2 and between cysteines 3 and 4 in the sequence. Should the mature protein have consecutive disulphide bonds, the product would contain the appropriate disulphide bonds and no corrections would be needed. Unless, correction by isomerisation activity of disulphide isomerase (DsbC) would be required. Based on this model and explanation, only proteins which contain non-consecutive disulphide bonds in their final folded structure would be vulnerable to incorrect disulphide bond formation, consequently necessitating the action of cell isomerase (Berkmen *et al*., 2007). DsbC is expressed in *E. coli* and other bacteria, and promotes rearrangement of erroneous disulphide bonds allowing generation of the proper array in the final folded product. DsbC, a homodimer protein containing an uncharged hydrophobic pocket with room to accommodate misfolded proteins fully translocated into periplasm, functions as a protein-folding chaperone. As misoxidized proteins are most likely misfolded, chaperone property of DsbC recognizes them (Berkmen *et al*., 2007; Darby *et al*., 1998). Nevertheless, the mechanism by which the subsequent steps in disulphide bond isomerisation by DsbC occur has not been fully uncovered.

DsbG is a second disulphide bond isomerase (245 amino acid sequence identity to DsbC) presented in the periplasm of *E. coli.* However, there are structural and biochemical differences in the peptide binding sites between DsbC and DsbG which can provide different substrate specificity and activity; for example, DsbG hydrophobic pocket is larger than DsbC one; and unlike the uncharged pocket of DsbC, there are seven negatively-charged amino acids within the pocket of DsbG. DsbG's function is not known in *E. coli* as no substrate for it has been identified (Andersen *et al*., 1997; Berkmen *et al*., 2007; Heras *et al*., 2004; Messens and Collet, 2006).

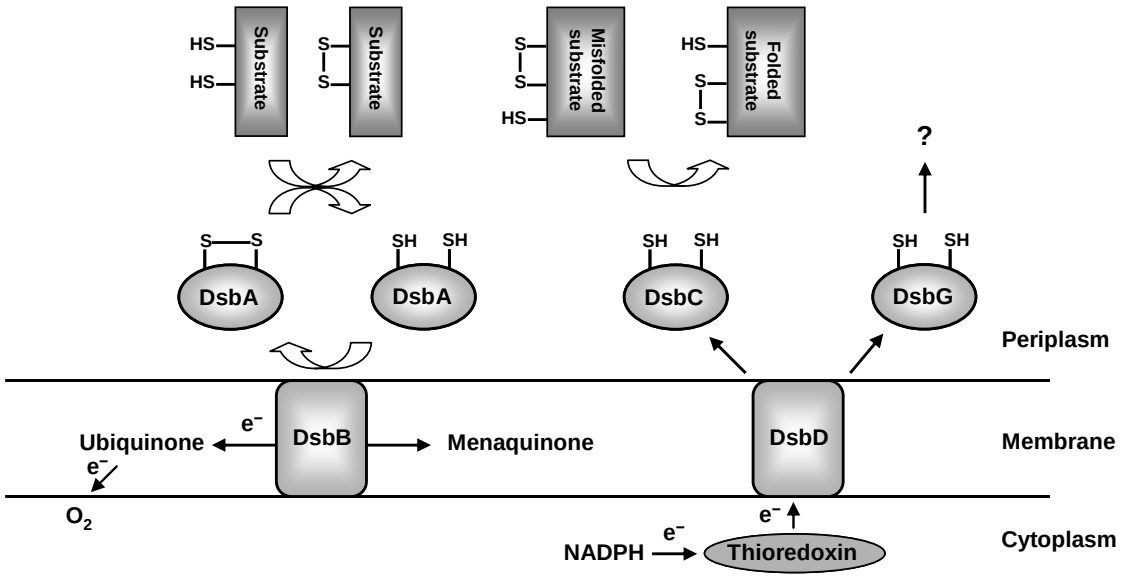

Fig. 1.4 Disulphide bond formation in the periplasm of *E. coli*. A protein requiring disulphide bonds for its stability is transported into the periplasm via the SecYEG translocon with its cysteines in a reduced state. Disulphide bond formation is catalysed by DsbA. DsbA is reoxidized to its active oxidized state by the inner membrane protein DsbB. DsbB is oxidized by ubiquinone in aerobic conditions or by menaquinone in anaerobic conditions. DsbA can form non-native disulphide bonds in proteins with more than two cysteine residues. If the substrate is misoxidized (misfolded), its disulphide bonds are isomerased to their native oxidized state by DsbC. DsbC and DsbG are kept in their active reduced forms by the inner-membrane protein DsbD. DsbD in turn is reduced by the cytoplasmic thioredoxin, which receives its reducing potential from cytoplasmic NADPH. (Figure adapted from Berkmen *et al*., 2007; Nakamoto and Bradwell, 2004).

DsbD, a 546-amino-acid cytoplasmic membrane protein, has been shown to be essential for keeping DsbC and DsbG in the reduced state (Bessette *et al*., 1999; Goulding *et al*., 2002). DsbD is reduced by the cytoplasmic thioredoxin TrxA receiving its reducing state from the cytoplasmic NADPH.

1.2.4 Periplasmic chaperones and isomerases

Biogenesis of myriad proteins necessitates the presence of molecular chaperones to ensure their efficient folding. The cellular task of chaperone molecules include keeping newly synthesized polypeptides from misfolding or aggregation, and promoting disaggregation and refolding of stress-denaturated proteins (Bukau, 1999). In gram-negative bacteria, the periplasmic and outer membrane proteins are synthesized in the cytoplasm with a cleavable N-terminal signal sequence and transported to their final destinations in the cell envelope using different pathways mentioned earlier. Following translocation and signal sequence cleavage, the exported extracytoplasmic proteins acquire their final structure via different folding pathways exist in the periplasm. Firstly, the periplasm of gram-negative bacteria assist the formation of disulphide bonds as described above; secondly, it contains ATP-independent chaperones assisting protein folding. Genetic studies of *E. coli* periplasmic stress response induced by increased amounts of non native envelope proteins led to the discovery of the periplasmic chaperones and folding catalysts (Table 1.2).

Table 1.2 Periplasmic chaperones and peptidyl-propyl isomerases (Table taken from Betton, 2007).

Protein	Chaperone activity	PPIase family	Oligomeric state	Transcriptional control
Skp (OmpH)	+		Trimer	Cpx, σ^E
PpiA (RotA)	-	Cyclophilin	Monomer	Cpx
FkpA	+	FKBP	Dimer	σ^E
SurA	+	Parvulin	Monomer	σ^E
PpiD	-	Parvulin	Monomer	Cpx, σ^{32}

Amongst the periplasmic folding proteins, Skp (OmpH) is the best-studied one, and scientists have shown that periplasmic Skp has a key role in the folding pathway of outer membrane proteins (Chen and Henning, 1996; Harms *et al*., 2001; Missiakas *et*

al., 1996). Although mutants lacking Skp have a markedly reduced production of outer membrane proteins, these mutants are viable; however, double mutants lacking both skp and the periplasmic Deg P can not survive at 37°C (Schäfer *et al*., 1999).
Periplasmic peptidyl-prolyl isomerases (PPIases) are enzymes catalyzing *cis-trans* isomerisation of peptide bonds with amino acid proline during protein folding (Betton, 2007). PPIases are categorized into three families, namely the cyclophilins, the FK506 binding proteins (FKBP), and the parvulins. Periplasmic proteins PpiA (or RotA), FkpA, and SurA and PpiD are respectively related to these three families of PPIases (Table 1.2). Like Skp, four PPIases are not individually essential for the viability in *E. coli*. Amongst PPIases, FkpA and SurA have chaperone activities as well.

In addition to Skp, FkpA and SurA, two periplasmic oxidoreductase , DsbC and DsbG, also show chaperone activities (Chen *et al*., 1999; Shao *et al*., 2000). It is assumed that these periplasmic folding catalysts can also perform chaperone activity to compensate the dearth of the molecular chaperone machineries in the periplasm (Betton, 2007).

1.2.5 Strategies for improving periplasmic protein accumulation

Biosynthesis of all proteins initiates in the cytoplasm.Therefore, for efficient periplasmic protein accumulation, events (such as translation initiation and protein folding) need to be coordinated. An example showing the relationship of cytoplasmic events and their effects on periplasmic export and yield was demonstrated by Simmons and Yansura (1996). They showed that that by altering the translational strength, through changes in the Shine Dalgarno sequence and the first six codons of the signal sequence without changing the actual primary protein sequence, the amount of protein accumulating in the periplasm was modulated. There appeared to be an optimal level of translation for efficient periplasmic accumulation without precursor buildup. High levels of the precursor may overwhelm cytoplasmic factor(s) required for maintaining the protein in an export-competent conformation. As a result of inadequate levels of such cytoplasmic factors, the precursor folding into an export-incompetent form or even forming inclusion bodies may occur. There are many

reports demonstrating the positive effects of certain cytoplasmic proteins on periplasmic indigenous and/or recombinant protein accumulation (Berges *at al*., 1996; Phillips and Silhavy, 1990; Wild *et* al., 1992, 1996). The molecular explanation is that the chaperonic properties of such cytoplasmic proteins (e.g., DnaK, DnaJ, and GroEL) prevent the target preprotein from premature folding and inclusion body formation, keeping the protein in an unfolded state and allowing efficient export to the periplasm.

Changing signal sequences is a common strategy to improve periplasmic protein production (Le Calvez *et al*., 1996; Rathore *et al*., 1996; Humphreys *et al*., 2000; Laird et al, 2004b). The alternative signal sequences could target the protein for post- or cotranslational export; they may interact differently with different cytoplasmic proteins; and they can cause changes in mRNA secondary structures that affect translational speed, mRNA stability, and mRNA translational strength, impacting the level of protein production.

A considerable percentage (in the 20 to 40% range) of the total cell protein can be exported to the periplasm (Joly *et al*, 1998; Jeong and Lee, 2001; Laird *et al*., 2005) when conditions for periplasmic accumulation are optimized. These figures have been reported without overexpression of the components of secretory pathways (i.e., Sec and Tat pathways). It has been shown that it is feasible to increase the efficiency of protein export into the periplasm of *E. coli* by providing the host with additional copies of key components of Sec protein export machinery (Perez-perez *et al*., 1994). Also, overexpression of the TAT export machinery has been achieved and elevated yields (over 20-fold) of recombinant proteins exported to the periplasm (Barrett *et al*., 2003).

The folding environment in the periplasm plays a key role in the final form of a secreted protein. Following export from the cytoplasmic membrane machinery, periplasmic proteins require to fold into their correct conformations and form protease-resistant structures. There are three possibilities for newly secreted upon release into the periplasm (Fig. 1.5). Firstly, proteins may missfold and aggregate

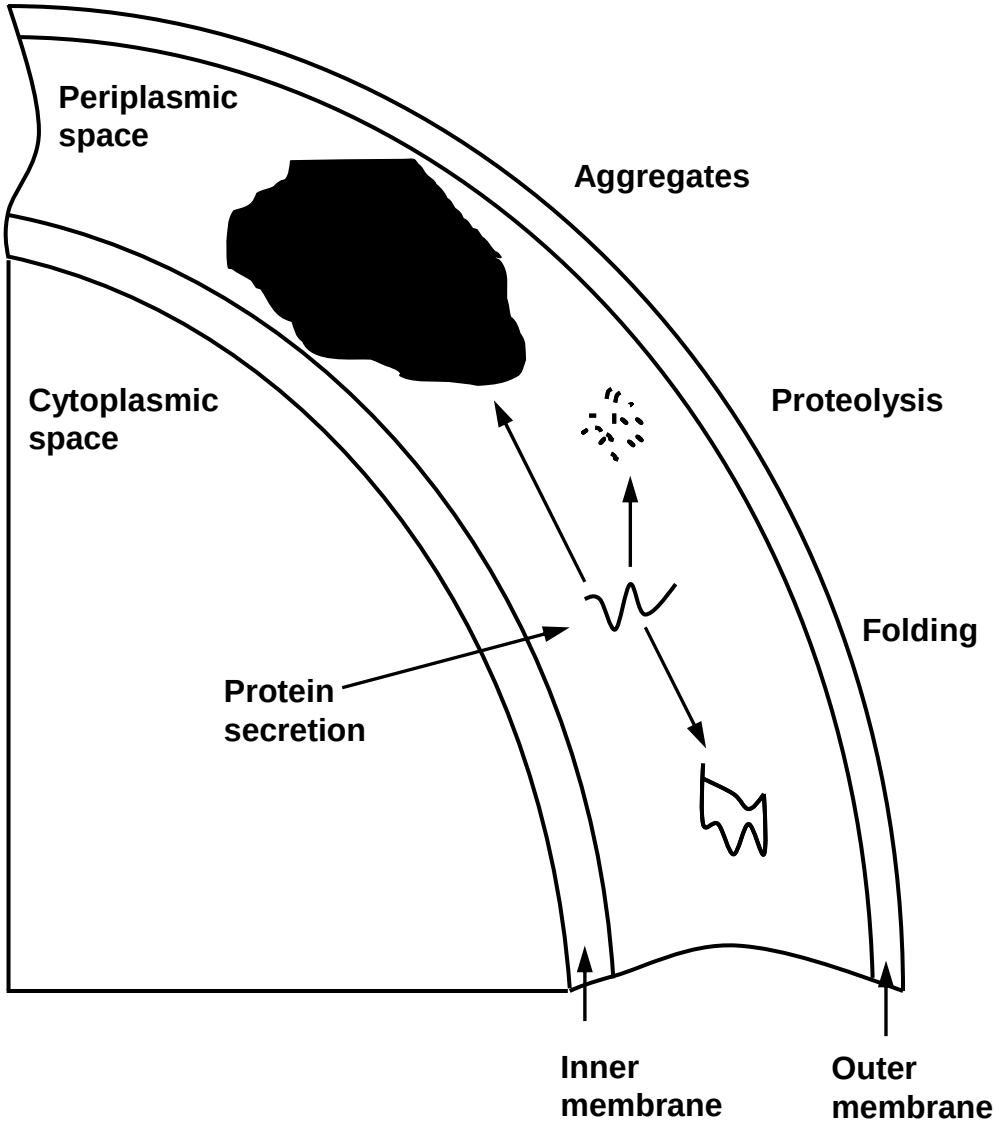

Fig. 1.5 Possible destinies of proteins after secretion into the periplasm (adapted from Joly and Laird, 2007).

which result in insoluble inclusion bodies (IBs). Aggregation occurs among intermediates with exposed hydrophobic patches, which are buried in the correctly folded protein (Carrio and Villaverde, 2002; Vallejo and Rinas, 2004) Secondly, proteins can be digested by periplasmic/membrane proteases. Finally, proteins can successfully fold into their correct soluble, biologically active conformations; this is usually preferred for those trying to exploit the periplasm as a location of recombinant protein production. The IB formation in the periplasm is not favourable as solubilising

and refolding of IBs are costly requiring many operations, as mentioned earlier in this chapter.

Elevated yields of recombinant protein production can be attained by other strategies which ere overexpression and deletion of certain periplasmic enzymes. The normal periplasmic level of the enzymes involved in disulphide bond formation and isomerisation, being sufficient for bacterial cells growth under normal laboratory conditions, can be inadequate for production of recombinant proteins. Many periplasmic proteins in *E. coli* do not contain more than two disulphide bonds; in contrast, eukaryotic proteins such as tissue plasminogen activators and immunoglobulin proteins contain great number of disulphide bonds (Simmons *et al*., 2002; Qiu *at al*., 1998) and boosting the periplasmic proteins involved in disulphide bond formation is necessary for efficient production of such proteins. Therefore, such enzymes have been overexpressed by many researchers to enhance the yield of periplasmic recombinant protein production (Bessette *et al*., 2001; Humpherys *et al*., 1996; Joly *et al*., 1998). Also, overexpression of proteins involved in the periplasmic folding process such as Skp and FkpA (Kleinschmidt, 2007), has been shown to augment the expression of periplasmic recombinant proteins, particularly the production of antibody fragments (Bothmann and Pluckthun, 1998; Hayhurst and Harris, 1999). As highlighted earlier, removal of deleterious periplasmic proteases is the other strategy for increasing yield of periplasmic recombinant protein production. This has been accomplished by obliterating the genes encoding periplasmic proteases without compromising the normal robustness and viability of the host (Baneyx and Georgiou 1990, 1991; Chen *et al*., 2004; Laird *et al*., 2004a). The removal of *ompT* gene, encoding OmpT protein, is frequently required for recovering periplasmically recombinant proteins without proteolysis. It is common to use hosts lacking *ompT*, such as *E. coli* B laboratory strain, BL21, to avoid such proteolysis (Joly and Laird, 2007; Laird *et al*., 2004a; Meerman and Georgiou, 1994). Furthermore, in order to increase recombinant protein production and to protect the protein production factory from extraneous assaults, such as bacteriophages, the genes encoding outer membrane protein (such as TonA, OmpC and OmpF), being bacteriophage receptors, have been eliminated without causing any detrimental growth defects (Hancock and Braun, 1976; Xiong *et al*., 1996).

In addition, there are also chemical approaches for improving protein folding; that is, the solute composition of the periplasm can be manipulated by regulating the medium composition. The outer membrane of gram-negative bacteria is permeable to solutes having molecular weight 600Da and less (Nakea and Nikaido, 1975). Thus, this could be utilized to improve protein folding by including components such as sucrose, glycerol, betaine, small thiol/disulphide reagents and etc., while overexpressing recombinant proteins in the periplasm. These solutes may help protein folding in the periplasm via triggering protective stress response attributable to their high osmolarity, or interacting directly with a folding intermediate (Barth *et al*., 2000; Blackwell and Horgan, 1991; Joly and Laird, 2007).

Eventually, the combination of overexpression of periplasmic components contributing in protein folding pathway and the inclusion of chemical additives has been reported to be considerably fruitful (Sandee *et al*., 2005; Wunderlich and Glockshuber, 1993).

As a major category of biotherapeutic proteins, antibodies are increasingly used in the clinic, and their importance is rising as more drugs in this class progress through clinical trials. *E. coli* is currently the host of choice for manufacturing antibody fragments in the periplasm due to the characteristics discussed above.

1.3 Antibodies and antibody fragments

Intact antibodies, or immunoglobulins, (IgG, IgM, IgA, IgE) are highly specific targeting reagents and provide our key defence against pathogenic organisms and toxins. Antibodies are able to selectively bind molecules, so called ‘antigens’ recognised as foreign to the organism. In 1930’s, antibodies were identified as the protective agent in the serum (Kuby, 1997), and the basic structure of immunoglobulins was elucidated during 1950’s and 1960’s by Rodney Porter and Gerald Edelman (Bak, 2004). ‘Polyclonal antibodies’ are a combination of immunoglobulin molecules identifying many antigen determinants or ‘epitopes’ on an antigen with a range of affinities (Bak, 2004), and these antibodies are usually produced by immunization of mammal, such as a mouse, rabbit or goat. Ploy clonal

antibodies have variety of applications. They can be exploited as a mean of inducing passive immunisation (e.g. for treatment against Hepatitis A, snakebites and tetanus); they are also extensively employed in diagnostics, immunoturbidemetry, and immunohistochemistry (Bak, 2004). 'Monoclonal antibodies', developed by Köhler and Milstein (1975), are identical antibodies identifying one epitope on antigen. Monoclonal antibodies can be produced by fusing antibody-excreting B-cells and myeloma (cancer) cells, the cell resulted in from this fusion being called hybridoma cell. Because of their single epitope specificity, monoclonal antibodies have wide applications in diagnostics, research, and therapeutics, ranging from *in vitro* pregnancy tests against the hCG hormone to *in vivo* cancer treatments (Bak, 2004).

IgG, the main serum antibody, is a Y-shaped molecule composed of 12 globular domains, named as Ig fold, that are rich in β-sheet and have a conserved disulphide bond (Humphreys, 2007). The Ig folds are connected by short interdomain linkers, named as elbow, or longer linkers known as hinges (Fig. 1.6). Most antibodies have two light chains and two heavy chains; each chain has a variable N-terminal region (fragment variable, Fv) known as V_L and V_H, respectively. Each variable region has three hypervariable loops (complementary determining regions, CDRs) that together form a common six-loop antigen binding site. The Fv region in each chain is followed by a different number of constant regions, as shown in figure 1.6.

Antigen-binding antibody fragments could be resulted in by proteolysis of whole antibodies. The protease papain digests the full-length IgG and releases the four N-terminal domains; the fragment produced is called the Fab fragment (fragment antigen binding), and has a molecular weight around 50 kDa. Pepsin cleaves the heavy chains slightly after hinge; thus, the dimeric Fab' fragment, or F(ab)', is produced (Fig. 1.7). Both enzymes release the C-terminal four domains or Fc fragment, which is glycosylated in the C_H2 domain and has effector functions such as serum half-life, complement activation, and recruitment of immune cells (Holliger and Hudson, 2005; Humphreys, 2007).

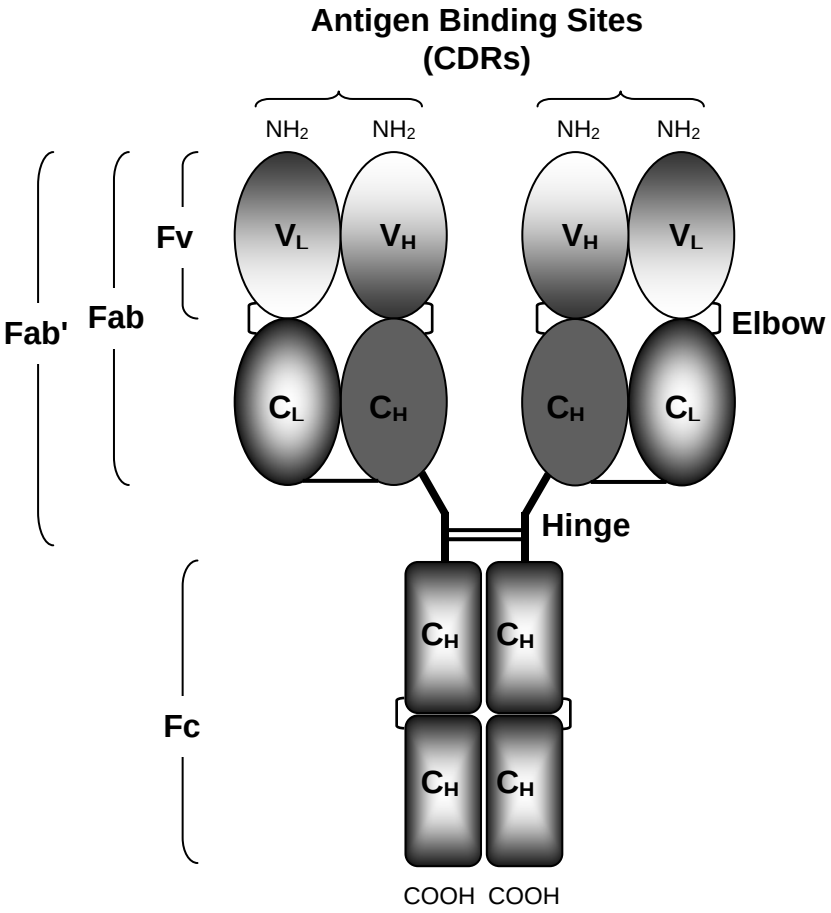

Fig. 1.6 Schematic representation of full-length IgG. Each heavy chain is composed of four domains: one variable (V_H) and three constant (C_H1, C_H2, and C_H3). Each light chain has two domains: one variable (V_L) and one constant (C_L). The two heavy chains are covalently attached by disulphide bonds between hinges and each light chain is attached to a heavy chain by a disulphide bond.

The minimal fragment (~30 kDa) that still contains the whole antigen binding site of a whole IgG antibody is composed of both the variable heavy chain (V_H) and variable light chain (V_L) domains. This heterodimer fragment, called Fv fragment (for fragment variable), is still capable of binding the antigen, but native Fv fragments are unstable since the non-covalently associated V_L and V_H domains tends to dissociate from one another.

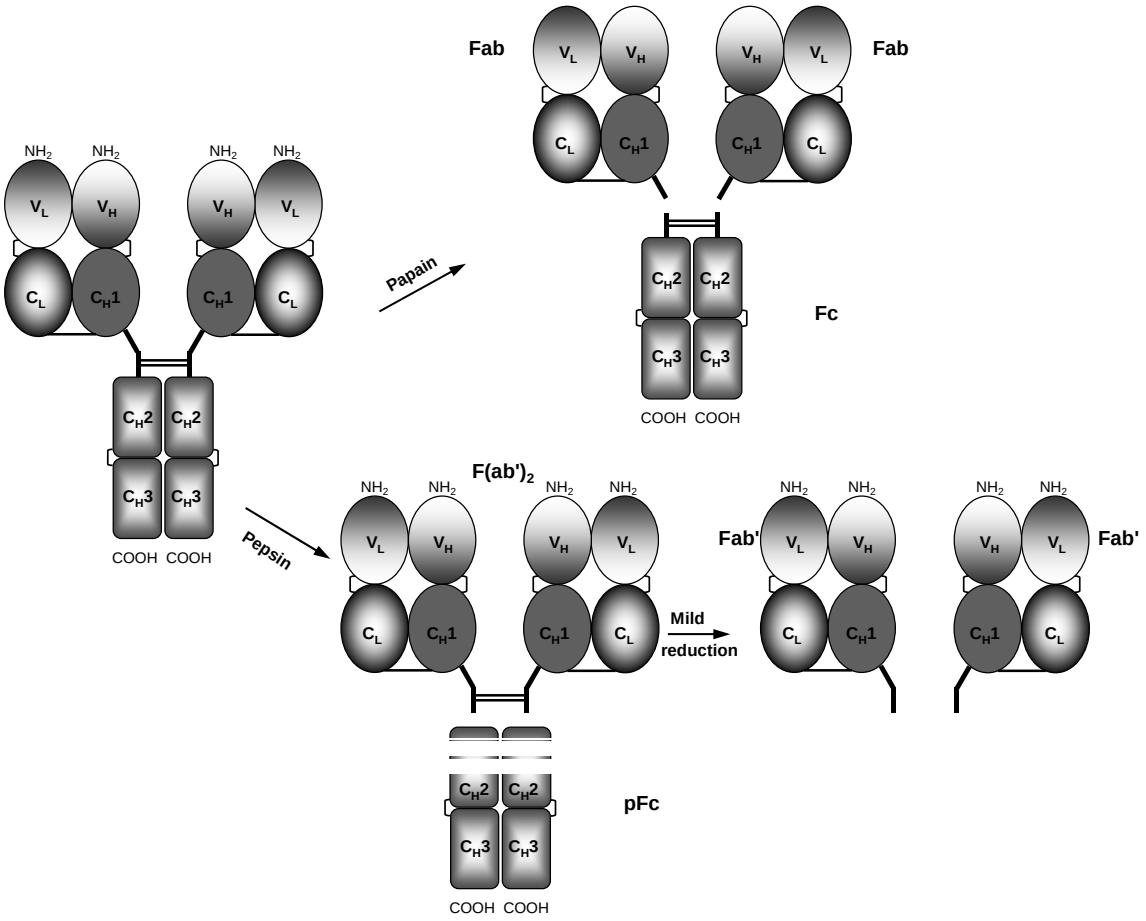

Fig. 1.7 IgG molecules can be digested with the enzymes papain or pepsin. Papain digestion results in cleavage of the immunoglobulin into Fab and Fc fragments, and pepsin digestion causes cleavage of the IgG molecule into F(ab)'$_2$. Pepsin also digests the Fc regions into fragments.

The production of antibody fragments in *E. coli* (e.g. Fab, Fv, scFv, V_Hs, diabodies, triabodies, etc.) has been successfully achieved (Holliger and Hudson, 2005; Humphreys, 2007 and references therein). These smaller fragments have altered features (e.g., monomeric binding, lack of effector functions, rapid systemic clearance, and rapid tissue penetration). However, it turned out to be difficult to produce fragments such as single domain antigen binding fragment (V_Hs) in soluble form, due to the sticky behaviour of the fragment which would cause aggregation. Such recombinant antibodies are increasingly used over a broad spectrum of

therapeutic and research applications. In the clinic, antibodies can be fused to species such as enzymes, viruses, radionuclides and toxins for drug therapy, cancer targeting, tumour and clot imaging (Hudson, 1998; Humphreys *et al*., 1997; Jurado *et al*., 2002; Natarajan *et al*., 2008; Wörn and Plückthun, 2001). In human medicine, a completely new approach, which is referred to 'magic bullet', is the fusion of an effector protein to an antigen recognising antibody fragment (Joosten *et al*., 2003). The gene encoding the effector protein can be directly fused to the gene of an antibody fragment, resulting in new bi-functional proteins (Neuberger *et al*., 1984). An example is the employemnet of cancer-specific bi-functional antibodies targeting cytotoxic molecules to tumour cells and consequently eliminates these tumour cells without harming healthy cells (Boleti *et al*., 1995). In the laboratory, antibodies can be used in immuonoblotting, enzyme-linked immunosorbent assay (ELISA), and affinity purification. The increasing demand for recombinant antibodies has been driving the development of a variety of production systems as diverse as yeasts, mammalian cells, transgenic plants and animals, and *Escherichia coli* (Andersen and Reilly, 2004; Hudson, 1998; Joosten *et al.*, 2003)). In comparison to other expression systems, *E. coli* has much to offer for low-cost and high-speed protein production, based on perfect familiarity with the genetics, physiology and regulatory systems of this organism; limitations for high-level expression in *E. coli* have been overcome by the engineering of the host. For example, there has been report on production of aglycosylated Fc-containing antibody in the periplasm of *E. coli* (Simmon *et al*., 2002). Although, it is deemed that manufacturing of full-length antibody is very difficult in *E. coli* because this organism is not especially well-adapted for the expression of very large, disulphide bond-rich and multimeric proteins. For many of the mentioned applications, the presence of glycosylation in the Fc constant regions is unnecessary or fruitless; thus, in these applications, aglycosylated full-length antibodies or smaller antibody fragments can be employed (Andersen and Reilly, 2004).

As a type of antibody fragments, the Fab fragment of an antibody contains the antigen-binding site and the four domains (i.e. V_H, C_H1, V_L and C_L); V_H and V_L domains are fused to the following constant antibody domains C_H and C_L, respectively. The smaller size of Fab is more suitable for applications such as tumour

penetration and imaging, and Fab exhibits a shorter half-life than full-length immunoglobulin G molecules (Andersen and Reilly, 2004), resulting in the faster clearance of the toxic labelled antibody fragments from the circulation in normal organ. However, the short circulating half-life of Fab fragment can be a drawback for therapeutic applications, but this constraint can be overcome by approaches such as attachment of polyethylene glycol (PEG) (Chapman *et al*., 1999; Chapman, 2002; Leong *et al*., 2001) and albumin-binding peptide (Dennis *et al*., 2002), which have been proved to increase the circulating half-life. In contrast to some of the heavily engineered antibody fragments, the Fab has native sequence; subsequently, it is less likely for the Fab to be immunogenic when used as therapy. The thermal ($\geq$ 60°C) and chemical ($\geq$ 2 M guanidine hydrochloride) stability of Fab make it resistant to strident bioprocessing and modification processes (Humphreys, 2007).

As mentioned earlier, there are three locations for the manufacture of soluble proteins in *E. coli*: the cytoplasm, the periplasm, and secretion into the culture media. The periplasm is preferable for expression of soluble antibodies, mainly because it facilitates the formation of disulphide bonds, the retention of correct N-terminus, and the exposure to extracellular chemical agents (<500 Da in size) which aid the folding and modification of antibodies (Humphreys, 2007). A diversity of approaches, including optimising the expression of light and heavy chains to obtain augmented titres of antibody fragments (Humphreys *et al*., 2002), host cell engineering to hinder proteolysis of the light chain (Chen *et al*., 2004), and co-expression of chaperones to ameliorate folding or disulphide bond formation (Ramm and Plückthun, 2000; Humphreys *et al*., 2002) have been implemented to enhance the periplasmic production level of antibody fragments up to 2 g/L using the *E. coli* system (Carter *et al*., 1992; Chen *et al*., 2004).

Having achieved desirable levels of expression in the periplasm, it is required to develop the extraction processes to release the maximize amount of the recombinant protein while minimizing the release of other host proteins and biomolecules. At large scale, the prevalent technique for purifying proteins from periplasm includes breaking the cytoplasmic membrane and the outer membrane and discharging the entire content of the bacterial cells. Inasmuch as approximately 80 per cent of the total

cellular protein is located in the cytoplasm, this method of non-selective- protein release makes the purification process burdensome (Joly and Laird, 2007). At laboratory small scale, the frequent methods for cell fractionation and isolating periplasmic fraction are spheroplasting and osmotic shock treatment (Neu and Heppel,1965), but these techniques are not practicable when culture volume reaches above 10 litres (Joly and Laird, 2007). Finding disruptions techniques which can selectively release periplasmic fraction at large scale and alleviate the subsequent protein recovery processes is still a goal to be achieved.

1.4 Agents permeabilising the outer membrane

As illustrated in figure 1.8, the cell wall of gram-negative bacteria consists of an outer membrane (OM) and a peptidoglycan or murein layer. Peptidoglycan layer composed of glycan chains consisting of N-acetylglucoseamine and N-acetylmuramic acid linked by β (1-4)-glycosidic bonds, and the glycan chains are crosslinked by formation of peptide bonds between amino acid side chains. The wall grants mechanical strength to the cell due to the crosslinked peptidoglycan layer, the thickness of which alters in different growth phases of the cell, in particular, over the course of transition from exponential phase to stationary phase. Stationary-phase bacteria have generally a thicker and more highly crosslinked peptidoglycan layer (Middelberg, 1995; Volmer, 2007).

The OM is a lipid bilayer containing transmembrane proteins (porins), lipoproteins, phospholipids and lipopolysaccarides (LPS). Lipoproteins join the lower section of the lipid bilayer to the peptidoglycan layer. The outer leaflet of the OM is believed to contain only lipopolysaccharides, which are crossbridged by divalent cations (e.g., Mg^{2+} and Ca^{2+}) and do not have a high degree of permeability to hydrophobic molecules. The inner leaflet of the OM consists of phospholipids (Vaara, 1992).

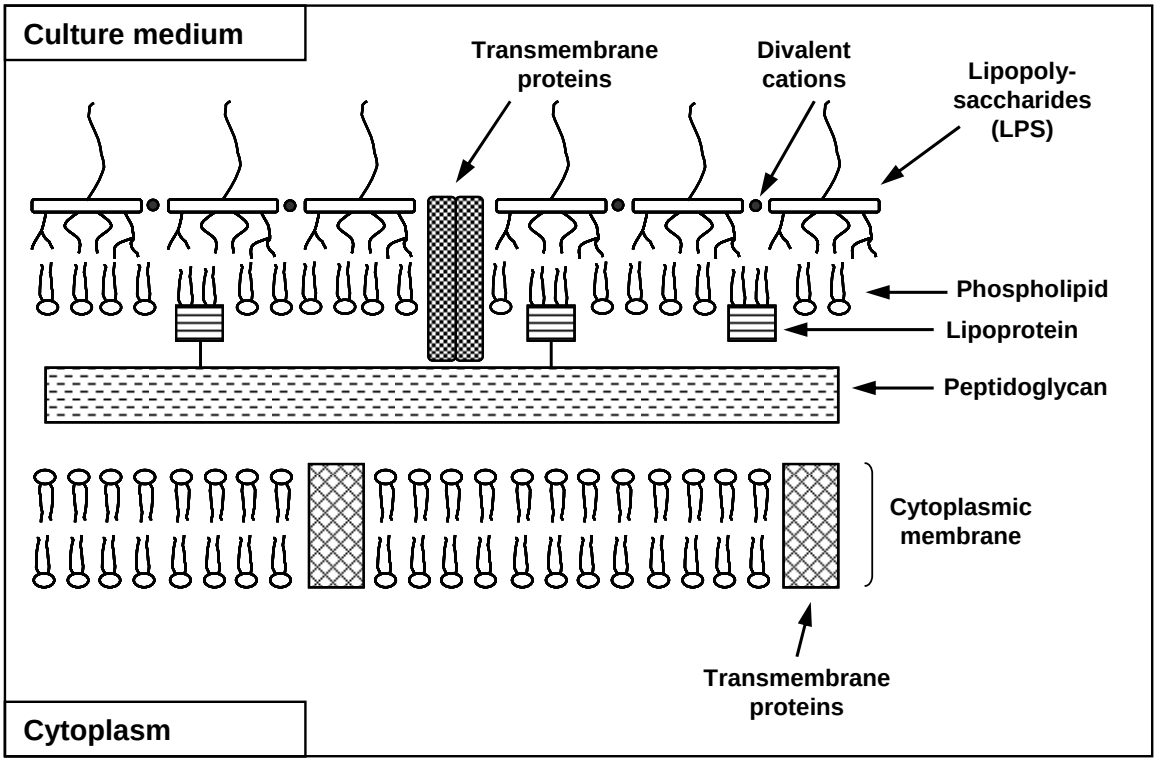

Fig. 1.8 Simplified structure of the cell wall of *E. coli* showing its relationship with the cytoplasmic membrane (redrawn from Middelberg, 1995).

It has been demonstrated that phospholipid bilayers are permeable to hydrophobic molecules, and bacteria in which the OM contains phospholipid bilayer regions (e.g., *Nisseria gonorrhoeae*) are more vulnerable to hydrophobic agents such as erythromycin, Triton X-100, and crystal violet (Lysko and Morse, 1981; Sarubbi *et al*., 1975). Therefore, the LPS layer in gram negative bacteria is deemed to protect the phospholipids in the inner leaflet of the OM and in the inner (cytoplasmic) membrane from pernicious agents such as neutral and anionic detergents, hydrophobic antibiotics, and hydrophobic dyes (e.g., brilliant green, methylene blue and eosine). Small hydrophilic compounds can diffuse through the OM via the water filled porin channels (Nikaido and Vaara, 1985).

Release of all intracellular components requires complete destruction of the cell wall, and it is usually achieved by mechanical techniques (e.g., high-pressure homogenization and bead milling); however, gentler disruption techniques, such as chemical and enzymatic methods, can be employed for this purpose. Chemicals interacting selectively with components of the cell wall and cell membrane may allow product to leak through the peptidogylcan; in which case, there is no need to raze the peptidoglycan layer. Otherwise, the disruption of petidoglycan in gram negative bacteria is needed, which generally involves enzymatic method (e.g., lysozyme) attacking the peptidoglycan layer (Middelberg, 1995).

Various chemicals (e.g., antibiotics, chelating agents, chaotropes, detergents, and solvents) can be applied, singly or in combination, in order to disrupt or permeabilise the OM of gram negative bacteria.

Cationic antibiotics (e.g., polymyxin), which are too large to go through the narrow channels of porins of the OM, can gain access to their final target in the cytoplasmic membrane of gram-negative bacteria via binding to LPS and thus disrupting the OM. It is also demonstrated that polymyxin could indeed enhance the permeability of the OM to many agents such as hdrophobic antibotics, detergents, and lysozyme (Middelberg, 1995; Nikaido, 2003; Nikaido and Vaara, 1985; Vaara, 1992). Despite of their ability to cause cell lysis, antibiotics can be used neither for large-scale lysis, as they are generally costly, nor for selective release of periplasmically-located proteins.

Chelating agents, such as EDTA, nitrilotriacetate, and sodium hexametaphosphate, have been demonstrated to permeabilise the OM of gram-negative bacteria by chelating Mg^{2+} and Ca^{2+}, which crossbridge adjacent LPS molecules, leading to the release of a considerable proportion of LPS from the cells (Hancock, 1984; Hancock and Wong, 1984; Vaara, 1992; Vaara and Jaakkola, 1989). The space generated by LPS release is most likely filled with phospholipid molecules from the inner leaflet of the OM; the created phospholipid bilayer patches can then function as channels through which hydrophobic compounds can diffuse.

The primary amine as well as an organic cation, Tris, not only synergistically assists the function of chelating agents, such as EDTA, but also singly and at moderately high concentration (e.g., up to 200 mM) can permeabilise the OM (Hancock, 1984; Nikaido and Vaara, 1985; Vaara, 1992). This is supposed to be attributed to Tris ability to interact with LPS which replaces stabilizing cations, and as a result weakens the interaction between LPS molecules. It has been shown that the impact of EDTA on LPS structure has been diminutive in the presence of buffers such as phosphate buffer and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), but strong in the presence of Tris. Hence, it can be construed that a maximal OM destabilization requires not only the removal of divalent cations, but also replacement of those and other cations by Tris (Middelberg, 1995; Vaara, 1992).

Chaotropes, such as urea and guanidinium chloride, disorganize the structure of water rendering it less hydrophilic, and weaken solute-solute interaction. Therefore, hydrophobic compounds such as membrane proteins can be solubilised in reasonably concentrated chaotropic solutions. Although chaotropes should usually be employed at high concentration while they use singly, effectual protein release has been reported at low chaotrope concentration (e.g., 0.1 M guanidinium chloride) in combination with non-ionic detergent Triton X-100, which can be fruitful for large-scale application of chaotropes for cell permeabilisation (Bansal-Mutalik and Gaikar, 2003; Hettwer and Wang, 1989; Middelberg, 1995). Ethanol, usually categorized as an organic solvent, is also an effective chaotrope inasmuch as it disrupts the hydrogen bonding in water (Middelberg, 1995).

Having an ionic hydrophilic region and a hydrophobic hydrocarbon portion and hence being amphipathic molecules, detergents (surfactants) are able to interact both with water and lipid to form micelles containing lipid. Detergents may be categorized as anionic (e.g., sodium dodecyl sulphate (SDS) and sodim deoxycholate), cationic (e.g., cetyltrimethylammonium bromide (CTAB) and benzalkonium chloride (BAC)), and non-ionic (e.g., Triton X and Brij series). A crucial parameter in choosing a detergent for permeabilisation purpose is its impact on the target product. For instance, while SDS causes protein denaturation in most cases, non-ionic detergents (e.g., Triton X-100) solubilise membrane proteins without a loss of activity

(Middelberg, 1995; Schnaitman, 1971). The outer membrane of gram negative bacteria is resistant to non-ionic detergents, and the recovery of cytoplasmic membrane proteins from pretreated cells (for example, with EDTA-lysozyme) by detergents proves the specificity of most non-ionic detergents for the cytoplasmic membrane (Burman *et al*., 1972; Filip *et al*., 1973). Cationic detergents operate on the OM by binding to the negatively charged LPS, and also modify the conformation of membrane proteins leading to cell disruption (Bansal-Mutalik and Gaikar, 2003). Given their specificity for the inner (cytoplasmic) membrane, it is liable that detergents continue to attract attentions for extraction and recovery of specific membrane proteins at process scale. Although it is implausible that detergents individually receive approval for whole cell disruption because of process cost and complicated downstream processing, it is likely that they will be considered to be employed at low concentration and in combination with other treatments (e.g., EDTA and chaotropes) for destabilizing the OM of gram-negative bacteria and cell disrupting (Hettwer and Wang, 1989).

Solvents, such as toluene, chloroform, ether, can also permeabilise cells, allowing soluble protein release. They most likely operate by dissolving hydrocarbon components, such as phospholipids, in the cell wall of gram-negative bacteria. An increase in release by solvents in the presence of chemicals destabilizing the OM (e.g., EDTA) has been reported. Also, it has been demonstrated that OM stabilizing agents, such as Mg^{2+} and Ca^{2+}, diminish the protein release by solvents. Therefore, the release by solvents relies on the selected conditions (Ames *et al*., 1984; Bansal-Mutalik and Gaikar, 2003; De Smet *et al*., 1978; Jackson and Demoss, 1965).

The final objective of all bioprocess production and recovery works is to obtain correctly-folded and biologically-active target biomolecules such as proteins. During various stages of biomolecules extraction and recovery, it is favourable to study the structures of the molecules. Circular dichroism, discussed below, is one of the most powerful tools for this purpose.

1.5 Circular dichroism and its applications

When circularly polarized light passed through an optically active substance, the left- and right-circularly polarized rays are absorbed to a different extent; that is, $\varepsilon_L \neq \varepsilon_R$. The difference $\Delta\varepsilon = \varepsilon_L - \varepsilon_R$ is called circular dichroism (CD) (Woody, 2000). For a quantitative and standard description of CD, the Beer-Lambert law is addressed.

In absorption spectroscopy the fraction of light absorbed by a sample follows the Beer-Lambert law:

$$A_\lambda = \log_{10} (I_0/I) = \varepsilon lC$$

Where A_λ is the optical density or absorbance, I_0 is the intensity of light entering the sample, C is the concentration of the sample given in moles L^{-1}, l is the path length of in cm and ε is the molar extinction coefficient and has units of litres $(moles\text{-}cm)^{-1}$ (Miles and Wallace, 2006; Woody, 2000).

In an optically active medium, this equation also applies in CD spectroscopy. In this case, CD is the difference in absorption of left- and right-circularly polarized light by optically active molecules; that is,

$$CD = \Delta A = A_L - A_R = \varepsilon_L lC - \varepsilon_R lC$$

Where the subscripts indicate the direction of rotation of light. CD instruments (known as spectropolarimeter) usually generate the data in mdegrees or ellipticity (θ) units (Woody, 2000; Miles and Wallace, 2006).

There is a numerical correlation between ΔA and ellipticity (in degrees), namely $\theta = 32.98\ \Delta A$. The CD spectrum is acquired when the “dichrograph” is gauged as a function of wavelength; this is the way a spectrum is stored in a computer (Kelly *et al*., 2005). Figure 1.9 illustrates a schematic diagram of a spectropolarimeter.

The salient optically active groups in proteins are the amide bond of the peptide backbone (absorption below 240 nm) and the aromatic side chains (absorption in the range 260 to 320 nm) (Greenfield, 1996; Kelly *et al*., 2005). As shown in Fig. 1.10, various secondary structures, such as α-helices and *β*-pleated sheets, have their own distinctive CD spectra, regarding shape and magnitude (Johnson, 1988).

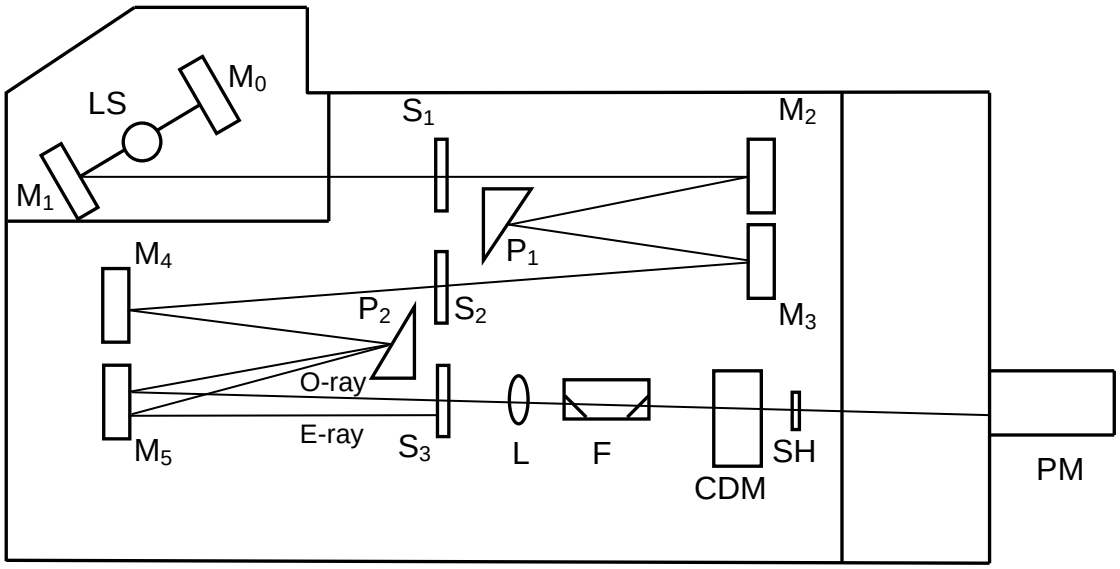

Fig. 1.9 Diagram of a spectropolarimeter (Jasco J-810). Passage of light from the source (LS) through 2 prisms (P_1 and P_2) and a series of mirrors (M_0 to M_5) and slits (S_1 to S_3) produces plane polarised light. After being focused by a lens (L), the ordinary ray (O) produces circularly polarised light by passing through a filter (F) and modulator (CDM). The circular polarised light is then passed through the shutter (SH) and the sample, and is detected by the photomultiplier (PM) (Figure redrawn from reference: Kelly *et al*., 2005).

The most effective region of the CD spectra is the far-UV region (approx. 240-190 nm). Conformational changes which involve modification to aromatic side chains of proteins can be monitored in the near-UV region (300-240nm); however, the CD signals in near-UV region are less intense (Wallace and Janes, 2001).

As it is shown in figure 1.10, the CD spectrum for an *α*-helix takes over the region between 240 and 200 nm, with two reasonably intense negative bands at about 222

and 208 nm. The CD spectrum of the *α*-helix also dominates the region between 200 and 174 nm, with a strong positive band at 192 nm. The CD spectrum derived from *β*-sheet is characterised by a small negative band near 217 nm and a fairly intensive positive band near 195 nm that has approximately half the intensity of the *α*-helix positive peak in this region. *β*-sheets give rise to considerably less intense signals than helices; show more variations in spectral characteristics, due to more vulnerability to distortions caused by the CD signals arisen from aromatic side

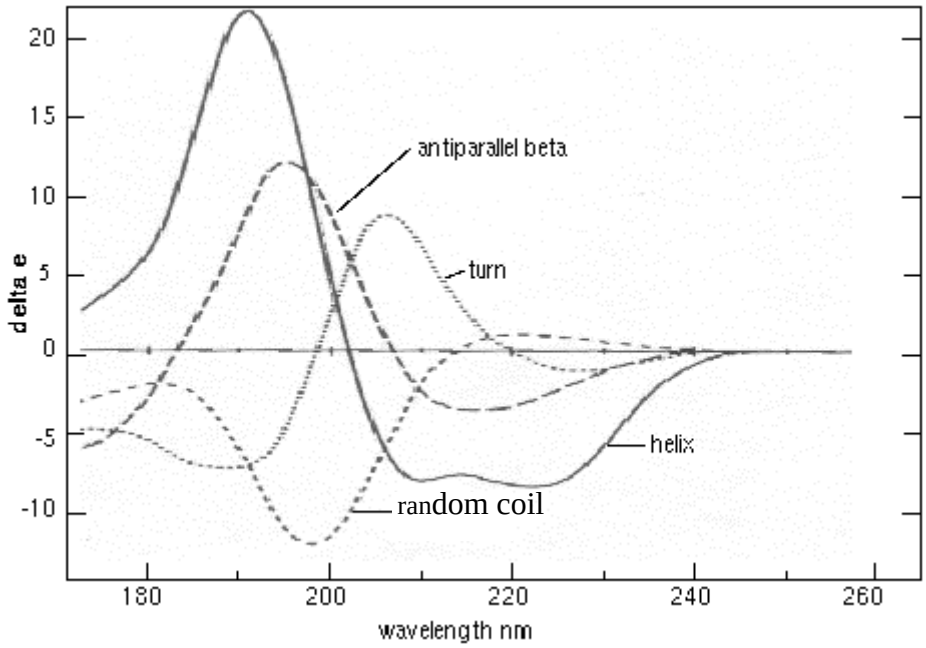

Fig. 1.10 Circular dichroism of “pure” secondary structures. (Figure taken from Johnson, 1988).

chains. Analyses of *β*-sheet rich proteins are thus less accurate than those of their helical counterparts (Miles and Wallace, 2006). The CD spectrum of random coil is identified by a small positive band between 215 and 230 nm and a strong negative band at 198 nm. The CD spectrum of polyproline (PPII) helix, the conformation which is found in collagen and in short segments of some globular proteins, is generally similar to the one arisen from random coil (also known as unordered or disordered structure (Greenfield, 1996; Miles and Wallace, 2006).

The foremost application of CD is for analysis of secondary structure, as the CD spectrum is susceptible to the small alterations in the secondary structure of proteins, and thus provides information on conformational alterations in proteins (Dafforn and Rodger, 2004; Johnson, 1988; Wallace and Janes, 2001), particularly while no data are available from X-ray technique. For instance, the CD spectra for 36 salt-extracted ribosomal proteins from *Escherichia coli* have been reported, denoting that 30S proteins tend to have a high content of *α*-helix, while the 50S proteins have very dissimilar structures (Johnson, 1988).

As the CD spectra in near-UV (260-320 nm) derive from the aromatic amino acids, thus this technique can be used for study of the tertiary structure of proteins. Each amino acid has a unique wavelength profile. Trp has a band between 290 and 305 nm, Tyr shows a band between 275 and 282 nm, and Phe has sharper bands between 255 and 270 nm (Johnson, 1988). The near-UV CD spectrum can be applied to compare wild type and mutant form of proteins; for example, this application has been reported for wild type and mutant (R23Q) type II dehydroquinase from *Streptomyces coelicolor* (Kelly *et al*., 2005; Krell *et al*., 1996).

CD can also provide information on cofactor binding sites in proteins. Organic cofactors, such as pyridoxal-5´-phosphate, heam and flavin, have weak CD signals when they are free in solution. Binding to their partner proteins, these cofactors confer chirality and thus CD spectrum in the specific region can indicate the integrity of the cofactor binding site. CD spectrum has been employed to provide structural information about haem site in cytochrome P450s and bacteriochlorophyl1 sites in LH_2 (light harvesting) complex from *Rhodopseudomonas acidophila* (Alden *et al*., 1997; Anderson and Peterson, 1995; Hope *et al*., 1996; Kelly *et al*., 2005).

There are numerous examples of the application of CD for studying protein structure, protein folding and protein conformational alterations. CD has been used for evaluating the structural relationship between wild type and mutant protein (Krell *et al*., 1996). Conformational modifications in proteins can be due to the binding of ligands, for instance, binding of molybdate to molybdate-sensing protein ModE from *E. coli*. In this case, CD data are invaluable for finding the range of ligand

concentrations over which structural changes occur, the extent of the changes in the protein of interest, and the rate at which such modifications take place (Boxer *et al*., 2004; Kelly *et al*., 2005). The switch between *α*-helix and *β*-sheet structures in prion peptides and the switch between helical hairpin and coiled-coli motifs in designed peptide sequence have been investigated using CD (Hope *et al*., 1996; Pandya *et al*., 2004). A particular type of structural change happens throughout the gaining of the native structure of a protein during biosynthesis, and CD has been employed to measure the rate of acquisition of secondary and tertiary structures. For example, it has been revealed that small proteins (fragments less than 100 amino acids) can fold rapidly with no detectable intermediates; whereas, the folding of larger proteins is a multi stage pathway at which intermediates, namely “molten globule”, are appeared (Jemth *et al*., 2004; Kelly *et al*., 2005).

Preparation of protein samples for CD studies should be done carefully. The important conditions for protein sample preparation have been discussed below.

1.5.1 Protein preparation, characterisation and storage

The majority of proteins are currently produced by over-expression of their relevant encoding genes in an appropriate host system (such as *E. coli*, yeast or animal cells). For conducting CD experiments, proteins should be free of optically active impurities, such as nucleotide fragments or optically active buffers and chemicals, which might largely contribute to the final CD spectrum (Greenfield, 1996; Kelly *et al*., 2005).

Sometimes in order to improve protein purification, a small tag (such as hexa-His) is attached to the protein encoding gene, or the protein of interest is expressed as a fusion protein with glutathione *S*-transferase (GST) or maltose-binding protein (MBP). These additional components provide a recognition site for purification using affinity chromatography. The tag should be obliterated using an appropriate protease if its presence influences the folding and stability of the protein. For instance, GST or MBP segments (26 kD and 40 kD, respectively) of fusion proteins must be eliminated before structural studies by CD spectroscopy, due to extensive contribution of these large moieties to the CD spectra of the fusion protein (Kelly *et al*., 2005) .

The protein solution should be also free from chiral components. Nucleic acids (or nucleotide fragments) should be eliminated by adding a suitable nuclease to the extract obtained from cell lysis before conducting protein purification.

Dialysis or gel permeation can be utilised in order to abolish buffer ions which might cause problems with CD. For example, imidazole employed to elute His-tagged proteins from immobilised metal chromatography (Ni-NTA) column, and chloride ions (NaCl) used to elute proteins from ion-exchange columns, show high absorbance in the far-UV and must be removed from the solution using dialysis or gel permeation (Kelly et al., 2005).

Protein solution should be transparent with no insoluble protein aggregates, inasmuch as these alter the shape and magnitude of the CD spectra and also decrease the signal/noise ratio (Kelly *et al*., 2005). Spectrophotometry and analytical ultracentrifugation (AUC) can be utilised to corroborate the presence of aggregates. In the spectrophotometric technique, the absorption spectrum of the protein sample is measured at the wavelengths generally ranged from 400 nm to 240 nm. If there is a gradually increasing baseline optical density as the wavelength decreases, this shows that light scattering exists. Sensitive to the mass and shape of the protein, sedimentation velocity experiments can be done using an analytical ultracentrifuge in order to monitor the aggregation of proteins (Berkowitz, 2006; Cole and Hansen, 1999; Liu *et al*., 2006). Protein solution can be centrifuged (e.g., at 5000×g, for 5 min) or passed through a 0.2 μm Millipore filter to reduce or eliminate aggregated materials or dust particles for CD studies (Kelly *et al*., 2005).

1.5.2 Selection of buffer/solvent system for CD measurements

A minimum ionic strength is usually needed to disperse the surface charges of the protein; suitable buffer systems are hence required. Buffers should be used within a range of approximately 1 pH unit on either side of the appropriate pK_a and should be prepared at a sufficient concentration to avoid changes in pH on addition of a highly charged ligand (Kelly *et al*., 2005).

Chloride ions are one of the worst interfering components. Chloride ions are part of phosphate buffered saline (PBS) or Tris buffers, in which HCl is used to adjust the required pH. Sodium chloride should be replaced by either sodium fluoride (unless this interferes with the protein structure or function) or sodium sulphate; In addition, Tris buffer should be acidified by using sulphuric or phosphoric acid instead of HCl. Anions such as sulphate or fluoride do not absorb significantly in far-UV (Kelly *et al*., 2005; Miles and Wallace, 2006).

Buffers such as HEPES, MOPS, MES and PIPES also absorb strongly in the far-UV; therefore, increasing the cut off wavelength (the lowest wavelength at which a spectrum can accurately be measured) and should only be used at low concentrations for detailed far-UV CD studies (Kelly *et al*., 2005; Miles and Wallace, 2006). The interfering problem also greatly occurs in the pH range 4 to 6, inasmuch as most of buffer such as citrate, acetate and glycine have carbonyl groups absorbing strongly in the far-UV (Kelly *et al*., 2005; Miles and Wallace, 2006). It should be mentioned that imidazole, which is employed at concentrations of 100 mM or above to elute His-tagged proteins from immobilised metal chromatography (Ni-NTA) columns, absorbs considerably in the far-UV; ideally, the concentration of imidazole should be reduced to lower than 1 mM (Kelly *et al*., 2005).

Moreover, protective agents; for instance salts (e.g., $(NH_4)_2\ SO_4$), osmolytes such as proline, sugars, sucrose, EDTA, protease inhibitors and dithiothreitol, can be included in the protein solution. By adding glycerol to 50% (v/v), the protein solutions can be stored at -20°C without being frozen. EDTA is frequently added as stabilizing agent because it chelates trace metal ions required for the action of some proteases, and also it chelates the metal ions which have detrimental effects on cysteine residue of the side chains. Addition of 1 mM EDTA does not significantly cause problem for CD experiments using a short path length cell (e.g., 0.02 cm), although the carboxylic groups of EDTA absorb below 200 nm. Dithiothreitol, reducing agent which maintains Cys side chains in their reduced state, absorbs significantly below 220 nm; hence, the absorbance of a buffer containing this agent should be meticulously tested in far-

UV region (Arakawa and Timasheff, 1982; Bolen, 2004; Kelly *et al*., 2005; Parkins and Lashmar, 2000; Wang, 1999).

Chemicals such as urea (8 M) and guanidinium chloride (6 M), which normally used for denaturation of proteins, greatly absorb below 210 nm. These chemicals at such concentrations do not permit reliable CD data to be collected below 210 nm, even by using cells of short path length (20 µm) (Kelly *et al*., 2005).

Detergents are usually applied to extract membrane proteins from their membrane milieu, the choice of which should be such as to sustain the structure integrity of the protein but should not cause excessive absorbance in the far-UV. Amongst commonly used detergents, Triton X-100 has high absorbance around 280 nm and it is difficult to remove from proteins. SDS is a useful detergent for CD studies because it lacks absorbance in the far-UV (Kelly *et al*., 2005).

Solvents containing carbon-chloride bonds to absorb significantly below 230 nm; dimethylsulphoxide and dimethyl formamide absorb strongly below 240 nm and 250 nm, respectively. Organic solvent such as acetonitrile ethanol and methanol (HPLC grade) can be applied down to 190 nm. 2,2,2-trifluoroethanol (TFE) is a useful solvent for studying peptides and protein fragments, having been demonstrated to enhance the dehydration of specific residues within a helix and as a result promote helix stability, can be used for CD studies (Kelly *et al*., 2005; Starzyk *et al*., 2005).

Overall, during CD measurements, it is important to maintain the total absorbance of the sample (i.e., protein and buffer or solvent) within rational ranges to avoid excessive noise. The appropriateness of any buffer/solvent system for CD should be checked by measuring blank spectra to ensure that the absorbance is not too high. The absorbance of the sample can be simply checked by tracing of High Tension (HT) voltage, which is the voltage applied to photomultiplier. HT should be generally less than 700 V for getting reliable data (Kelly *et al*., 2005). In general any buffers/solvents that absorb significantly in the wavelength range of interest should be removed or substituted with a non-absorbing buffer. In some cases, this drawback can be resolved by applying low concentration of buffers and short path length (50 to

100 µm) cells. Phosphate buffer systems are preferable, generally being used at concentrations up to 100 mM in cells with path lengths of less than 50 µm (Miles and Wallace, 2006). For measurements below 195 nm, in particular, it is vital to employ transparent buffers, such as 10 mM potassium phosphate (Greenfield, 1996). For measurements in low wavelength (below 170 nm), samples may also require to be degassed immediately before measuring a spectrum to eliminate dissolved oxygen (Miles and Wallace, 2006).

1.6 Aims and outline of the thesis

The broad objective of this thesis has been to design improved new processes for extraction and purification of periplasmically expressed proteins. Three periplasmically expressed proteins, namely beta-lactamase, alpha-amylase, and D1.3 Fab fragment, were selected as model proteins for the studies in this thesis. Antibody Fab fragments are more useful than full-length immunoglobulin G molecules in diagnostic applications, tumour therapy and tumour imaging. The smaller size of antibody Fab fragments causes rapid tissue penetration and the shorther half-life of the molecules results in rapid clearance from the blood or kidney. Due to these features, the production of Fab fragments is industrially highly demanded; and several Fab fragments are in clinical and preclinical development (Holliger and Hudson, 2005). Successful production of soluble and correctly folded Fab fragments in the periplasm of *E. coli* has been achieved. *E. coli* CLD048 produces Fab D1.3 (first model protein chosen for this study) in the periplasm, and it has been shown that the amount of periplasmic production of the Fab is higher at 25 and 30 degrees (information received from Shagayegh Arasteh). Fab D1.3 is anti-lysozyme antibody fragment; therefore, lysozyme can not be used in the cold osmotic shock to digest the peptidoglycan layer of the bacterial cell wall; therefore, partial recovery of the Fab is expected. At the very beginning of this study (Chapter 2), experiments were conducted to compare osmotic shock and mechanical cell disruption methods for the recovery of this ‘difficult-to-release’ antibody fragment (Fab) from the recombinant *E. coli*, and to show how product titre, location and form varied during fed-batch cultivation. Chapter 3 of this thesis describes purification of D1.3 Fab using affinity and ion exchange chromatography (IEC) techniques; the

purified Fab was used in further structural studies (Chapter 4). In Chapter 4, the effects of various concentrations of a large number of cell permeabilisation reagents on the secondary structure of three different pure ‘periplasmic’ target proteins (i.e. beta-lactamase, alpha amylase and Fab D1.3) were studied by a newly developed high throughput circular dichroism (ht-CD) system. The model proteins used in the study contain differing amounts of α-helix and β-sheet to aid the resolution of the data interpretation later, as *β*-sheets give rise to considerably less intense signals than helices, and show more variations in spectral characteristics (Miles and Wallace, 2006). The impact of cell permeabilisation chemicals on the biological activity of the model proteins was studied and correlations between the CD results and activity were drawn. Reagents classified as ‘safe’ in the chapter 4 study were subsequently evaluated in ‘periplasmic release’ tests (Chapter 5) conducted with *E. coli* cells harbouring the same target species.

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2 Fed-batch cultivation of *E. coli* CLD048 and production of anti-lysozyme Fab D1.3

Abstract

The bacterium *E. coli* CLD048 produces Fab D1.3 which is an anti-hen egg white Lysozyme (HEWL) antibody fragment. Fed-batch cultivation for production of Fab D1.3 was carried out at 37°C, and the bacterial cells were induced by adding 0.1 mM IPTG to the culture medium. The vessel was sampled over the course of fermentation and the bacterial cells were disrupted by osmotic shock (with excluding HEWL) and sonication. Amount of soluble Fab D1.3 in different locations (i.e. the culture broth, the periplasm, and the cytoplasm) was quantified during fed-batch cultivation. It was revealed that Fab D1.3 could be directed to different sites (i.e within and outside the cells) soon after induction. It was divulged that the Fab was saliently located in the cytoplasm and the periplasm 2 h after induction. After 2 h post induction, the Fab increasingly leaked into the culture medium, and reached its maximum culture medium titre of ~78 mg/L after 6 h post induction. After 16 h cultivation (6 h post induction) the amount of Fab remained constant in different locations within and outside the cells, and the culture medium was the place where the majority of the soluble Fab was located in. Western blot analysis of different cell fractions showed that a considerable amount of the Fab was produced in the cells as insoluble form.

2.1 Introduction

Antibodies and their fragments have been found to be of huge academic, clinical, and commercial value. Several new antibodies and antibody fragments (such as Fab, scFv, V_Hs, diabodies, triabodies) have been designed using recombinant DNA technology (Humphreys, 2007). Antibody fragments, lacking the glycosylated Fc constant regions, have the advantage over whole antibodies in applications requiring rapid tissue penetration and rapid clearance from the blood or kidney (Yokota *et al*., 1992). These features make antibody fragments useful in diagnostic applications, tumour therapy and tumour imaging (Holliger and Hudson, 2005; Wu and Senter, 2005). Development of bifunctional antibodies, through fusing the gene of antibody fragment to the gene of an effector protein (e.g. cytokine), have been a new approach for eliminating tumour cells without harming healthy cells (Boleti *et al*., 1995, Neuberger *et al*., 1984; Ortiz-Sánchez *et al.*, 2008). Usually, a systematic high dose administration of cytokines (e.g. interleukin-2, granulocyte macrophage colony stimulating factor (GM-CSF), tumour necrosis factor (TNF)-α, and interleukin-12) is required to obtain a concentration in the tumour microenvironment that is capable of activating an immune response, and this frequently cause devastating toxic side effects in the cardiovascular and respiratory systems (Dela Cruz et al., 2004; Ortiz-Sánchez et al., 2008). The use of bifunctional antibody-cytokine fusion proteins recognizing a tumour associated antigen reduces prolonged systematic cytokine exposure, resulting in less side effects of the drug towards healthy tissue.

The Fab fragment of an antibody contains the antigen-binding site and the four domains (i.e. V_H, C_H1, V_L and C_L). The heavy and light chains are covalently attached by a disulphide bridge, and V_H and V_L domains are fused to the following constant antibody domains C_H and C_L, respectively (Humphreys, 2007). The smaller size of Fab is more suitable for applications such as tumour penetration and imaging, and Fab exhibits a shorter half-life than full-length immunoglobulin G molecules (Andersen and Reilly, 2004). The short circulating half-life of Fab fragment can be occasionally a drawback for therapeutic applications, and this constraint can be overcome by approaches such as attaching polyethylene glycol (PEG) (Chapman *et al*., 1999; Chapman, 2002; Leong *et al*., 2001) and albumin-binding peptide (Dennis

et al., 2002), which have been proved to increase the circulating half-life. In contrast to some of the heavily engineered antibody fragments, the Fab has native sequence; subsequently, it is less likely for the Fab to be immunogenic when used as therapy. The thermal ($\geq$ 60°C) and chemical ($\geq$ 2 M guanidine hydrochloride) stability of Fab make it resistant to strident bioprocessing and modification processes (Humphreys, 2007).

As an expression system, the bacterium *Escherichia coli* has been the host of choice for the manufacture of many antibody fragments because its physiology and genetics has been known and it is therefore easily accessible for genetic modifications, and it requires simple inexpensive media for rapid growth. Over expression of recombinant proteins in the cytoplasm of *E. coli* may lead to the formation of inclusion bodies (IBs) within the cell which can contain the target protein in an misfolded form (Carrió and Villaverde, 2002; Heebøll-Nielsen *et al.*, 2003; Ventura and Villaverde, 2006). Manufacturing a protein by an IB route is very costly, due the large number of operation units needed for product recovery (Singh and Panda, 2005). Production hosts have been genetically manipulated to gain more oxidizing environment in the cytoplasm needed for disulphide bond formation and appropriate protein folding; nonetheless, it has been unproven whether high level (hundreds of milligrams per litre) of protein production can be achieved in such strains (Besette *et al*., 1999; Derman *et al*., 1993; Prinz *et al*., 1997). The periplasm, on the other hand, has been shown to be a much better site for the production of large quantities of soluble recombinant proteins, and promoters and secretion signals from a few outer membrane proteins have been used to direct recombinant proteins into the periplasm (Carter *et al.*, 1992; Chen *et al.*, 2004; Joly *et al.*, 1998; Laird *et al.*, 2005; Moir and Mao, 1990). The oxidizing environment of the periplasm in cooperation with the periplasmic disulphide oxidoreductases/isomerases (Berkmen *et al*., 2007) and periplasmic chaperones (Betton, 2007) assists the formation of correctly folded recombinant proteins. Also, the periplasm can be manipulated by being exposed to extracellular chemicals assisting protein folding (Barth *et al.*, 2000; Sandee *et al.*, 2005; Wunderlich and Glockshuber, 1993).

A common feature of many *E. coli* secretion systems is that the product may appear in many various locations within and outside the cells. In this chapter, experiments were carried out to show how anti-lysozyme Fab D1.3 titre, location, and form varied over the course of fed-batch cultivation. Also experiments were done to compare the efficiency of osmotic shock and mechanical cell disruption methods for the recovery of the antibody fragment from *E. coli.*

2.2 Materials and Methods

2.2.1 Materials

The tetracycline resistant *E. coli* CLD048 harbouring pAVE046 (Hodgson *et al.*, 2007), expressing the antibody fragment Fab D1.3 (secreting as heavy and light chains into the periplasm) was provided by Merck Sharp and Dohme Ltd. (Billingham, UK).

Nutrient agar and phosphate buffered saline (PBS) were purchased from Oxoid (Basingstoke, Hampshire, UK). Yeast extract and trypton were obtained from Becton, Dickinson and company (BD) (Sparks, MD, USA). Sodium dodecyl sulphate (SDS) and Laemmli electrophoresis and running buffers were attained from Bio-Rad Laboratories (Hercules, CA, USA). Ammonium hydroxide; phosphoric acid; magnesium sulphate; calcium chloride dehydrate; manganese (II) sulphate fourhydrate; and HPLC grade methanol were purchased from Fisher Scientific (Loughborough, Leicestershire, UK). TMB microwell peroxidase substrate (2-C) and TMB membrane peroxidase substrate system (3-C) were obtained from KPL (Gaithersburg, MD, USA). Hybond-P PVDF membrane was obtained from Amersham Biosciences (Uppsala, Sweden). Bicinchoninic acid (BCA) protein assay kit and Isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Thermo Scientific (Rockford, IL, USA). Tetramethylethylenediamine (TEMED) was supplied by Melford Laboratories (Suffolk, UK). SimplyBlue Safe Stain and SeeBlue Plus2 Pre-Stained Standard were purchased from Invitrogen (Paisley, UK). Ammonium sulphate; glycerol; potassium phosphate monobasic and dibasic; citric acid; ferrous sulphate heptahydrate; zinc sulphate; sodium molibdate dehydrate; copper (II)

sulphate; boric acid; tetracycline; sodium chloride; sodium hydroxide; Tween 20; ethylenediaminetetraacetic acid (EDTA); sucrose; Trizma-base; sodium phosphate monobasic; sodium phosphate dibasic; sodium carbonate; sodium hydrogen carbonate; glycine for electrophoresis; chicken egg white lysozyme (HEWL); albumin from bovine serum (BSA); and anti-human IgG (Fab specific) - peroxidise antibody produced in goat, as well as all other chemicals used in this study were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2.2 Methods

2.2.2.1 Fed-batch fermentation

Bacterial cells from the cell bank, stored at -80°C, were propagated overnight on Nutrient agar supplemented with 15 mg/L tetracycline. Starting cultures were prepared by inoculating 100 mL aliquots of Luria Bertani (LB) broth (comprising 1% NaCl, 0.5% yeast extract and 1% tryptone) containing 15 mg/L tetracycline with a fresh single colony of *E. coli* CLD048 and shaking at 37°C and 200 rpm for 13 h. A complex medium recommended by Merck Sharp and Dohme Ltd. (Table 1) was employed for the growth of *E. coli* in the fermentor. Fermentation was conducted using a 10 L Electrolab fermentor (Tewkesbury, UK) equipped with 6-bladed paddle-type impellers and four baffles located around the edge of the vessel. The vessel containing 5 L medium (Table 2.1) was sterilised at 121°C for 0.5 h and allowed to cool; post sterilisation and the trace metal additions (Tables 2.1 and 2.2) were then added. The pH of the medium was adjusted to 7.0 using ammonium hydroxide. The fermentor was inoculated with 200 ml (~4% inoc./medium ratio) from a 13-hour shake flask inoculum, and operated in batch mode. The starting conditions were: temperature 37°C; agitator speed 250 rpm; air-flow rate 1.0 VVM; pH 7.0. Agitation rate was increased during the fermentation (up to maximum value of 1500rpm), in order to sustain the dissolved oxygen tension on a set point of 30%. The pH was maintained at 7.0 throughout the fermentation by the automatic addition of NH_4OH. Once glycerol was depleted, being indicated by an increase in the dissolved oxygen measured in the fermentor (7.5 h cultivation), the glycerol/magnesium sulphate feed (Table 2.3) was initiated at a constant rate of 11.0 ± 2.0 (g glycerol/L/h). Fab D1.3

Table 2.1 Culture medium composition, the shaded part indicates the components which aseptically added after sterilisation and cooling of the medium to 37°C prior to inoculation of the fermentor.

Component of medium	Concentration required
$(NH_4)_2\ SO_4$	14 g/L
Glycerol	35 g/L
Yeast extract	20 g/L
KH_2PO_4	2 g/L
K_2HPO_4	16.5 g/L
Citric acid	7.5 g/L
Conc. H_3PO_4	1.5 mL/L
1 M $MgSO_4.7H_2O$ solution	10 mL/L
1 M $CaCl_2.2H_2O$ solution	2 mL/L
Antifoam AF204	0.2 mL/L
15 mg/mLTetracycline	1 mL/L

Table 2.2 Trace metal composition, 34 mL solution was sterilised through a 0.22 µm sterile filter into a suitable sterile container and added into 1 L medium in the fermentor.

Component	Concentration required
$FeSO_4.7H_2O$	3.36 g/L
$ZnSO_4.7H_2O$	0.84 g/L
$MnSO_4.H_2O$	0.51 g/L
$Na_2MoO_4.2H_2O$	0.25 g/L
$CuSO_4.5H_2O$	0.12 g/L
H_3BO_3	0.36 g/L
Conc. H_3PO_4	48 mL/L

Table 2.3 Glycerol and magnesium sulphate feed.

Component of medium	Concentration required
Glycerol	714 g/L
1 M Mg $SO_4.7H_2O$	30 mL/L

expression was induced by the addition of IPTG to a final concentration of 0.1 mM after 10 h cultivation (OD_{600nm}~50). The fermentor was sampled hourly; cell fractionation was made by sonication and osmotic shock, as described below, and various fractions were stored at -20 ^{o}C for further SDS-poly-acryl amide gel electrophoresis (SDS-PAGE), enzyme-linked immunosorbent assay (ELISA) and Western blot analyses.

2.2.2.2 Biomass estimation and viability counts

Optical density (OD_{600nm}) measurements were performed at a wavelength of 600 nm in a KONTRON UNIKON 922 UV-VIS spectrophotometer (KONTRON Northstar Scientific, Bardsey, UK), using PBS for dilution of cultures (readout range of 0.2 to 0.8 OD_{600nm} units).

Dry cell mass was measured as follows: 1 mL fermentation samples were centrifuged in pre-dried and pre-weighed 1.5 mL Eppendorf tubes using a Eppendorf microcentrifuge (Eppendorf Cenrifuge 5415D, Eppendorf, Hamburg, Germany) operated at 15,800 g for 5 minutes. The cell pellets were resuspended in 1 mL distilled water and then re-centrifuged. The supernatants were carefully discarded, the pellets were dried overnight in a hotbox oven (Gallenkamp, Riley Industries Ltd, Aldridge, UK) at 100°C, and the tubes were weighed once again. The biomass content in each tube was then calculated by difference.

Serial dilutions of fermentation samples were prepared aseptically in PBS for viability counts. One hundred microlitres aliquots of serial dilutions were plated onto LB agar plates supplemented with 15 mg/L tetracycline, and following overnight incubation at

37°C, the number of colony forming units (CFU) was measured by counting colonies on plates containing less than 300 colonies according to following formula.

$$CFU/mL = No.\ of\ colonies \times dilution\ factor \times 10$$

2.2.2.3 Cell fractionation

2.2.2.3.1 Osmotic shock

Cell pellets obtained from 1 mL fermentation samples were resuspended in an osmotic shock solution (designated OS_1) comprising 20 mM Tris-HCl buffer, pH 8.0, supplemented with 2.5 mM EDTA and 20% sucrose. After a static incubation on ice for 600 s, the cells were harvested by centrifugation (15,800 g, 120 s) and the supernatants were immediately frozen at -20°C. The resulting cell pellets (spheroplasts) were then resuspended in a second osmotic shock solution (designated OS_2) lacking sucrose (i.e. 20 mM Tris-HCl buffer pH 8.0, containing 2.5 mM EDTA) and incubated on ice for 600 s. Following centrifugation (15,800 g, 120 s), the supernatants were then removed and stored at -20°C until required. The pellet was washed with a high salt buffer as described later.

2.2.2.3.2 Sonication

Cell pellets obtained from 5 mL fermentation samples were resuspended in 5 mL of 100 mM Tris-hydrochloride buffer, pH 8.0. The resulting cell suspensions were then disrupted at 4°C by ultrasonication (ultrasonic liquid processor XL, Heat System Incorporated, Farmingdale, USA) at 55% amplitude for 300 s (15 cycles, each cycle lasted 10 s with intervals of 10 s between the cycles) to release intracellular protein. Successful cell disruption was confirmed with the aid of a phase contrast light microscopy. The bacterial cell debris was separated from the supernatants by centrifugation at 15,800 g for 300 s. The supernatant was frozen at -20°C for further analyses, and the pellet was used for high salt washing step as described bellow.

2.2.2.3.3 Washing the cell pellets obtained from osmotic shock and sonication cell disruptions with high salt buffer

The cell debris obtained from osmotic shock and sonication cell disruptions were respectively resuspended in 5 mL and 1 mL of 20 mM sodium phosphate buffer supplemented with 0.5 M NaCl, pH 8.0. The suspensions were agitated for 1 h and centrifuged at 15800 g for 300 s. The samples were stored at -20°C for further analysis.

2.2.2.3.4 Protein extraction from bacterial cell pellets

Cell pellets were resuspended in 500 µL Tris/EDTA/SDS buffer (10 mM Tris-HCl, 1 mM EDTA, 1% (w/v) SDS, pH 8.0) by mixing in a vibrax shaker (Clifton Cyclone, Scientific Laboratory Supplies, Notingham, UK) for 600 s followed by incubation at 80°C for 0.5 h. The supernatant was recovered by centrifugation at 15,800 g for 600 s, and total protein was measured using BCA assay employing BSA as standard.

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2.2.2.4 Analytical techniques

2.2.2.4.1 SDS-poly-acrylamide gel electrophoresis (SDS-PAGE) and Western blotting

SDS-PAGE was performed on 15% gels at 150V/20mA for 2.5 using Laemmli electrophoresis and running buffers. Prior to electrophoresis, all samples were diluted 1:1 with the sample buffer, boiled for 120 s, and then centrifuged briefly at high speed in a microcentrifuge, before loading into the wells of the gel. SeeBlue Plus2 Pre-Stained Standard used for estimation of molecular weight of protein bands on the gel. Following electrophoresis, gels were rinsed three times with 100 mL distilled water and then stained, with sufficient SimplyBlue Safe Stain (~ 20 mL) to cover the gel, for 1 h at room temperature with gentle shaking. The stain was discarded and the gel was subsequently washed twice with 100 mL distilled water for 1 h with gentle shaking.

For Western blotting, the stacking gel was removed and the resolving gel was soaked in the protein transfer buffer (3.03 g/L Trizma-base, 14.4 g/L glycine and 200 ml/L methanol) for 0.3 h. Hybond-P PVDF membrane and the filter paper were cut to the dimension of the gel, soaked in 100% (v/v) methanol for 10 s, washed in distilled water for 300 s, and equilibrated in the protein transfer buffer for 0.3 h. The electroblotting cassette was assembled and placed between the electrodes in the blotting unit according to the manufacturer's instructions (Mini Trans-Blot electrophoretic Transfer Cell, Bio-Rad, CA, USA). The band-transfer was carried out initially for 1 h at 100 V, with cold transfer buffer at 4°C, and was then perpetuated at 4°C, at 30 V, overnight. Following transfer, the membrane was removed from the blotting cassette, the orientation of the gel on the membrane was marked, and the membrane was rinsed briefly in phosphate buffered saline (PBS; 11.5 g/L di-sodium hydrogen orthophosphate, 2.96 g/L sodium dihydrogen orthophosphate, and 5.84 g/L sodium chloride; pH 7.5). For immunodetection, non-specific binding sites on the membrane were blocked using 3% (w/v) BSA made in PBS, for 1 h, and then briefly rinsed twice with PBS containing 0.1% (v/v) Tween 20. The blot was washed with an excess volume of PBS/0.15 (v/v) Tween 20 for 300 s and incubated with anti-human IgG (Fab specific) - peroxidise antibody (which was diluted down in PBS 10-fold) for 1 h. The blot was washed three times with PBS/Tween 20, and the enzyme substrate (TMB membrane peroxidise substrate system (3-C)) solution was added to the blot and incubated at room temperature until the desired band intensity was achieved.

2.2.2.4.2 Sandwich ELISA method for Fab D1.3

For this purpose, each well of a 96-well microtiter plate was coated overnight at 4°C with 100 µL of 0.1% HEWL, made in coating buffer (1.59 g/L Na_2CO_3 and 2.93 g/L $NaHCO_3$). Blocking was made with 200 µL PBS-BSA (1 PBS tablet/1 g BSA in 100mL distilled water) per well, with shaking (500 rpm) in a microplate incubator shaker (Infors AG type AK120, Infors HT, Bottmingen Switzerland) for 1 h, at 37°C. The wells were then washed three times with 300 µL wash buffer (1 PBS tablet and 100 µl Tween 20 in 100 mL distilled water) per well and tap dried. Both standards and samples (100 µL), having been diluted in PBS on a separate plate, were transferred to the washed ELISA plate, incubated at 37°C, 500 rpm for 1 h, washed

again with the wash buffer three times and tap dried. The plate was then loaded with 100 µL anti-human IgG (Fab specific) - peroxidase antibody produced in goat, which was diluted 10000-fold in the block buffer, incubated at 37°C, 500 rpm for 1 h, and then washed three times with the wash buffer and tap dried, before 100 µL of peroxidase substrate was added to each well. The reaction was stopped after 600 s by adding 100 µL of 1 M H_3PO_4 to each well, and the plate was read at the wavelength of 450 nm using a microplate reader (Promega Glomax-Multi detection system, Turner BioSystems Inc., Sunnyvale, CA, USA).

2.3 Results and Disscusion

During fed-batch fermentation, various parameters including OD_{600nm}, CFU, DCW, were measured, and these are presented in Fig. 2.1. It is evident from this figure that optical density reached the plateau after 13 h cultivation (3 h post induction with 0.1 mM IPTG), and started to reduce after 16 h cultivation (6 h post induction); however, there was a drop in CFU/mL after 2 h induction. Ideally, when cells are healthy, it is expected that both turbidity and CFU/mL follow each other. In this work, there was approximately 10-fold drop in CFU/mL for little increase in OD_{600nm} between 2 and 5 h induction. The existence of 'viable but nonculturable' bacterial cells can be the reason for the CFU/mL drop after 2 h induction with 0.1 mM IPTG. The term 'viable but nonculturable' has been used for those bacterial cells which have detectable metabolic function, but do not undergo cell division on routinely employed bacteriological media (Roszak and Colwell, 1987). Sundstorm *et al*. (2004) studied a process for the production of the recombinant fusion protein, promegapoietin (PMP), in *E. coli* and reported that after induction the number of CFU/mL dropped to ~10% of its maximum while the biomass concentration continued to increase. By doing flow cytometric analysis and measuring intracellular concentration of PMP, they demonstrated that the cells were alive and contributed to the production, and the drop in the number of CFU/mL indicated a loss of cell division ability rather than cell death. Also, in the shake flask studies done at 37°C on *E. coli* CLD048, it was reported that after 1 h induction with 0.1 mM IPTG (at OD_{600nm} = 10) there was a 10-fold drop in CFU/mL for a little increase in OD_{600nm}, and by flow cytometric analysis it was shown that this drop was due to the cells having broken cytoplasmic membrane

potential and as a result unable to recover and propagate on nutrient agar culture medium (Want, 2010). DCW figure was constantly increased during 16 h fed-batch cultivation, and afterwards there was an insignificant rise in this figure to the end of fermentation.

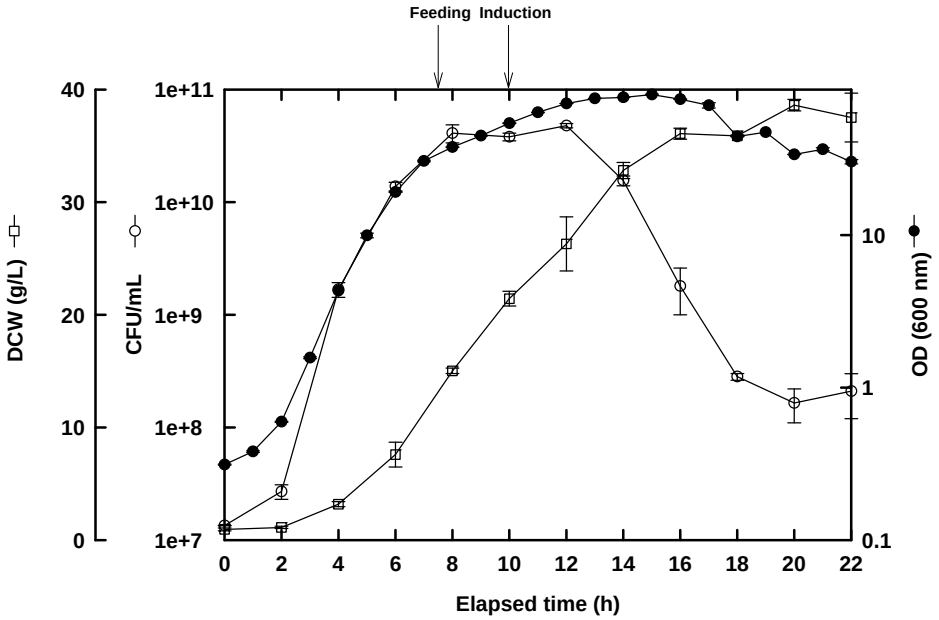

Fig. 2.1 Fed-batch fermentative production (initial volume 5 L) of Fab D1.3 (anti-lysozyme) in recombinant *E. coli* at 37°C. Induction with 0.1 mM IPTG was performed after 10 h of cultivation (OD ~50). Results for CFU and OD are averages of two measurements; results for DCW are averages of three measurements.

Two fractionation methods, including osmotic shock and sonication, were utilised to disrupt the bacterial cells present in the samples taken through the course of fed-batch fermentation. Subsequent cell disruptions, the titre of soluble Fab D1.3 in each fraction was quantified using ELISA. Figure 2.2 shows the titre of Fab in the culture broth medium and in the osmotic shock solution 1 and 2 (OS_1 and OS_2) at the point

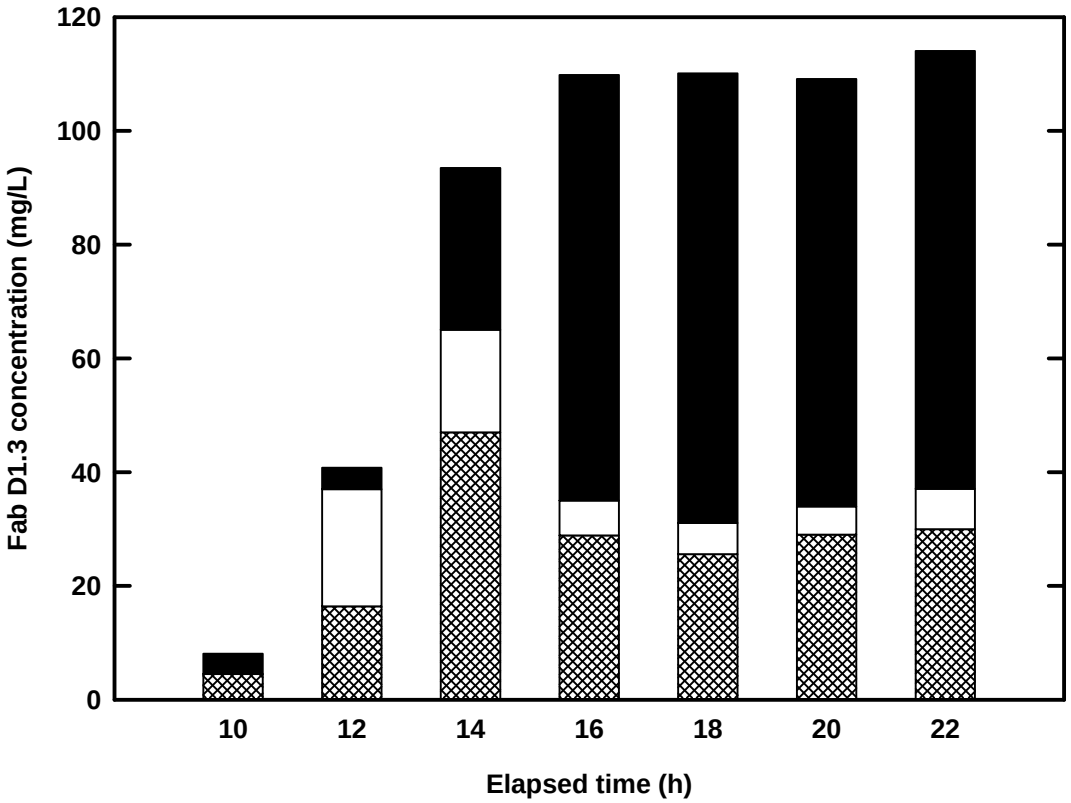

Fig. 2.2 ELISA of soluble and insoluble samples arising from osmotic shock fractionation. Induction was done by 0.1 mM IPTG after 10 h cultivation (OD~50). Amount of Fab quantified in osmotic solution 1 (▩), osmotic solution 2(□), and culture broth(■).

of induction and later. The existence of Fab D1.3 in OS_1 corroborates the periplasmic production of the protein, while the Fab titration in OS_2 shows the amount remained in the cytoplasm of the bacterial cells. As illustrated in the figure, a low amount (approximately 4 mg/L) of the Fab was observed in the periplasm and in the culture broth just before induction with 0.1 mM IPTG (i.e.10 h cultivation), indicating that the promoter was induced to an insignificant extent in the absence of the real inducer, i.e. IPTG. The amount of Fab D1.3 in the periplasm increased after induction and reached a maximum titre of 47 mg/L after 4 h post induction. The Fab secretion and/or leakage into the culture broth was sharply augmented after 2 h post induction, reached a maximum of about 79 mg/L after 6 to 8 h post induction, and stopped afterwards. Besides after 6-8 h post induction, the Fab titration in either periplasm or

cytoplasm remained almost constant, indicating that soon after the bacterial cells become unhealthy, based on OD_{600nm} measurements shown in figure 2.1, both cellular production and discharge of the recombinant protein are stopped in the cells. Based on these preliminary experiments, it is noticeable that if in a downstream processing the objective is to purify the secreted protein from the culture broth medium, there appears no benefit in perpetuating the fed-bath cultivation for a longer time (more than 16 h), like the one conducted for 35-45 post induction by Merck Sharp and Dohme Ltd. (Hodgson et al, 2007), as according to these results there is no further recombinant protein release into the culture broth and no product production in the cell after 6-8 h post induction.

The pellet samples left after osmotic shock cell disruption were washed with a high salt buffer (sodium phosphate buffer containing 0.5 M NaCl, pH 8.0), to dissociate any electrostatically-bond Fab from the pellet, and centrifuged.

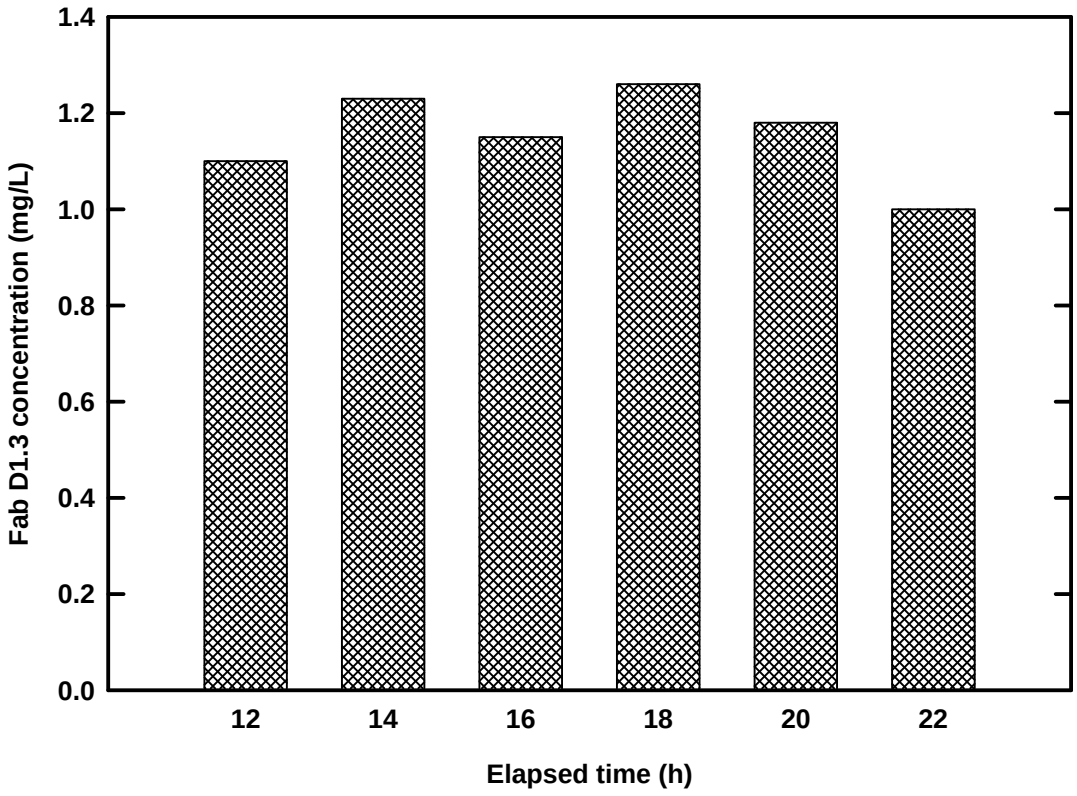

Fig. 2.3 ELISA analysis of soluble fractions obtained from washing post-osmotic shock bacterial cell debris with PBS supplemented with 0.5 M NaCl, pH 8.0.

ELISA analysis of the resulted soluble fractions showed that about 1.2 mg/L Fab D1.3 released by this washing procedure (Fig. 2.3).

Western blot analysis of these high salt-washed pellet samples, and of supernatants derived from OS_1 and OS_2 treatments have been shown in Fig. 2.4. As illustrated in this figure, it is evident that a fraction of Fab D1.3, as insoluble form, was in connection with the bacterial cell pellet shortly after induction (2 h post induction) with 0.1 mM IPTG. The Western blotting figures respective to the soluble samples (i.e. CB, OS_1 and OS_2) were generally in agreement with the ELISA ones (i.e., Fig 2.2), and demonstrated that there was an increasing amount of the Fab production and release into the periplasm and culture broth, respectively.

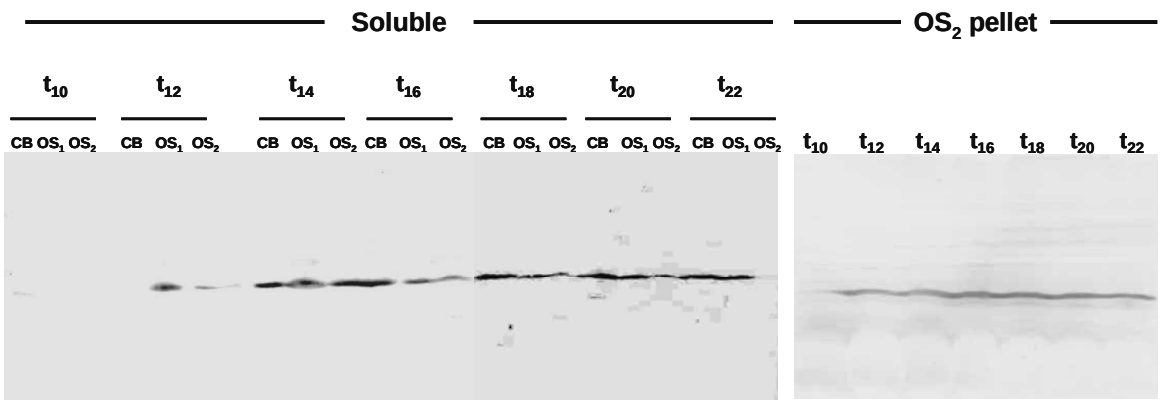

Fig. 2.4 Western blot analyses of soluble and insoluble samples arising from osmotic shock fractionation. Prior to Western blotting, the samples were subjected to non-reducing SDS-PAGE. Induction was made with 0.1 mM IPTG after 10 h cultivation (OD~50). Insoluble samples derived from osmotic shock were washed in a high salt buffer (sodium phosphate buffer containing 0.5 M NaCl, pH 8.0), centrifuged, and the obtained pellet samples were analysed.

Cell disruption by sonication was also performed on the samples taken from the fermentor throughout fed-batch fermentation. This mechanical cell disruption experiment was conducted in order to measure the amount of the Fab released by

this technique, and to compare the efficiency of two different cell disruption methods for the Fab release from bacterial cells. ELISA results for soluble samples obtained from sonication disruption have been illustrated in Figs. 2.5. By comparing ELISA results of osmotic shock and sonication disruptions, it is evident that the amount of Fab recovered from the bacterial cells by sonication was approximately 20 to 45% less than Fab recovered by osmotic shock (i.e. OS_1 plus OS_2) treatment (see Fig 2.2). This evinced that sonication had, most likely, deleterious impact on the Fab, afflicted its ability to attach to HEWL being coated onto ELISA plate. Therefore, lacking deleterious effects of sonication, osmotic shock seems to be a more effectual method for Fab recovery from the bacterial cells.

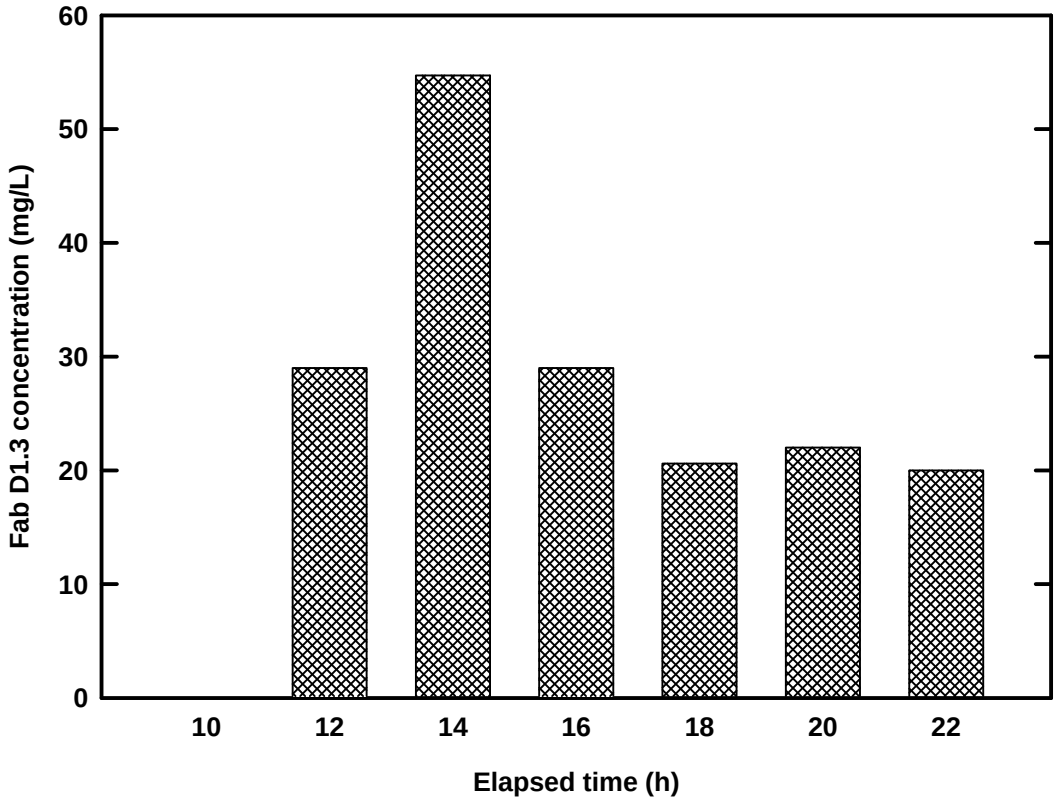

Fig. 2.5. ELISA of soluble samples arisen from sonication cell disruption. Induction with IPTG was after 10 h cultivation (OD~50).

Sonication cell disruption samples were also analysed by Western blotting (Fig 2.6). Pellets, obtained from centrifugation of samples after sonication, were washed with a

high salt buffer (sodium phosphate buffer containing 0.5 M NaCl, pH 8.0), to detach any electrostatically-attached Fab, centrifuged, and the resulting insoluble fraction were analysed by Western blotting. Western blot analysis of these samples showed that a considerable amount of the Fab was existed as insoluble form after sonication and high salt washing procedure.

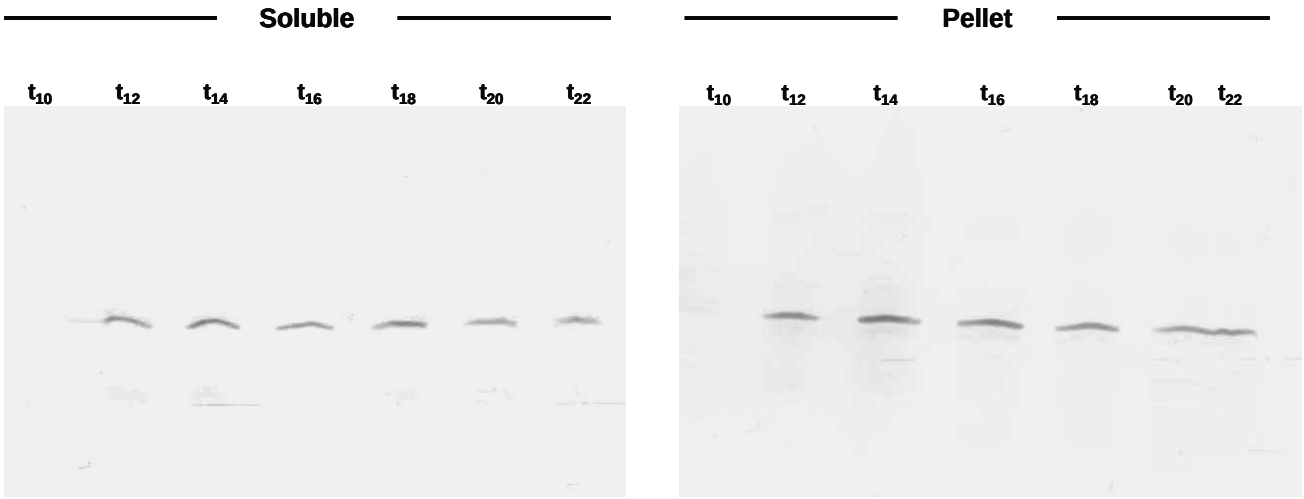

Fig. 2.6 Western blot analyses of soluble and insoluble samples arising from sonication cell disruption. Prior to Western blotting, the samples were subjected to non-reducing SDS-PAGE. Induction with 0.1 mM IPTG was after 10 h cultivation (OD~50). Insoluble samples derived from sonication treatment were washed in a high salt buffer (sodium phosphate buffer containing 0.5 M NaCl, pH 8.0), centrifuged, and the obtained pellet samples were then analysed.

There was no increase in quantity of the Fab in different fractions (i.e. culture broth, OS_1, OS_2, and supernatant of sonication) between 18 and 22 h cultivation; therefore, the samples taken after 22 h cultivation were not subjected to cell disruption procedures. Instead, only the bacterial cells in the sample taken from the fermentor after 58 h (i.e. the end of fed-batch fermentation) cultivation were treated by osmotic shock and sonication. ELISA results for this fractionation have been illustrated in Fig. 2.7, denoting that the amounts of Fab D1.3 in different fractions after 21 h and 58 h cultivation were the same. Also in this experiment, samples subjected to osmotic

shock treatment (i.e. OS_2 pellets) were disrupted by sonication. it was demonstrated that no or very inconsiderable soluble Fab remained in the bacterial cells after osmotic shock treatment, since sonication following OS_2 treatment did not release the Fab from the cell debris, of course with ignoring the portion of the recombinant protein possibly razed by sonication. This indicates that all soluble Fab fractions can be very effectively released from the cells by osmotic shock.

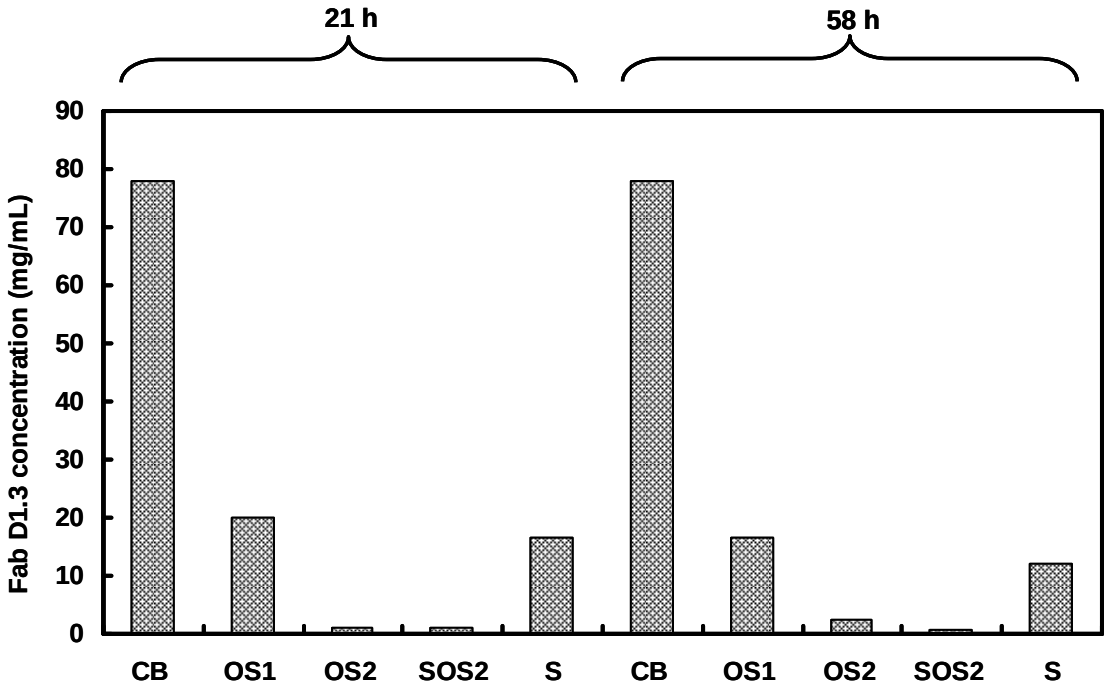

Fig. 2.7 ELISA analyses of soluble samples from osmotic shock and sonication treatments originating from 21 h and 58 h cultures. CB: culture broth, OS1: osmotic shock solution 1, OS2: osmotic solution 2, SOS2: sonication after OS2 and S: sonication.

Measuring total protein extracted from cell pellets arisen from osmotic shock and sonication treatments (sample 21 h) showed that with increasing the number of cell disruption routes less protein remained in connection with the cells, (Fig. 2.8); for instance, less protein remained in the cells when sonication followed OS_2 treatment.

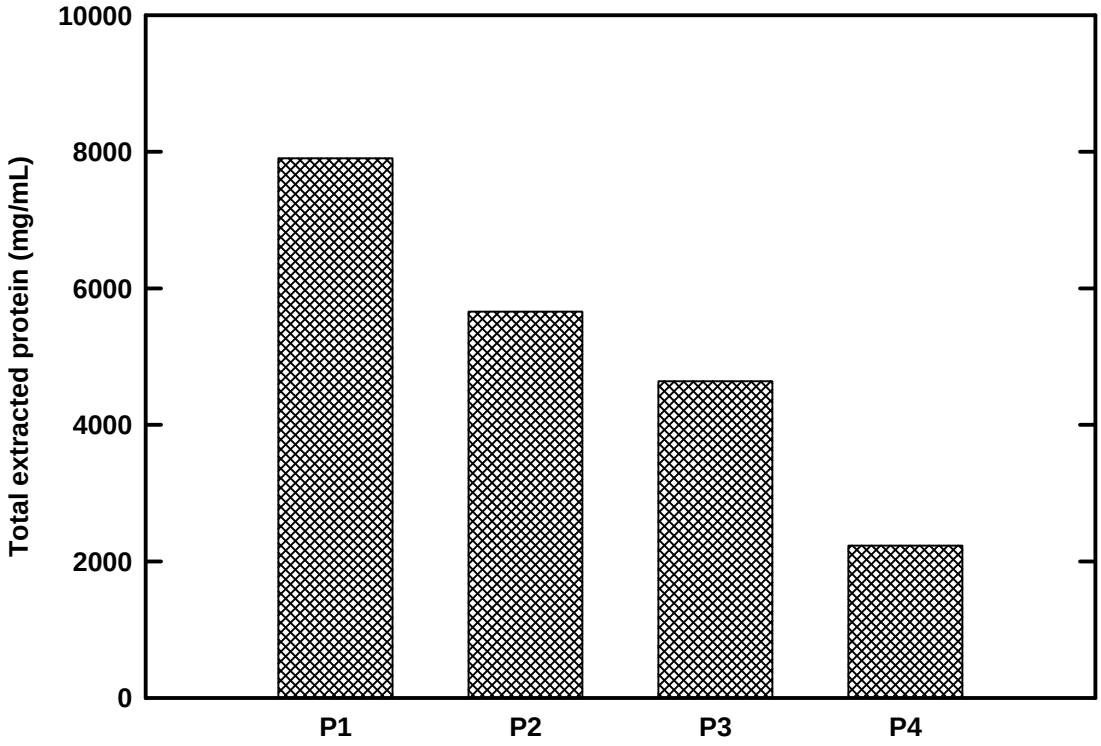

Fig. 2.8 BCA analyses of insoluble samples from osmotic shock and sonication treatments originating from a 21 h culture sample. Protein was extracted from the pellets by solubilisation with an EDTA/SDS/Tris-HCl cocktail at 80°C for 0.5 h as described in materials and methods. P1: pellet of intact cells, P2: pellet of sonication, P3: pellet of OS2, P4: pellet of sonication after OS2.

2.4 Conclusions

Production of anti-lysozyme Fab D1.3 during fed-batch cultivation of *E. coli* CLD048 at 37°C has been investigated. After induction with 0.1 mM IPTG, Fab D1.3 appears in different locations inside and outside the bacterial cells. Fab D1.3 in the periplasm reaches its maximum amount after 14 h cultivation (4 h induction with 0.1 mM IPTG). After 16 h cultivation, the majority of the Fab is appeared in the culture broth, and Fab titration remains constant in the cytoplasm, the periplasm, and the culture medium. D1.3 Fab is also produced in insoluble form and in attachment to the cell pellet as confirmed by cell fractionation with osmotic shock and sonication.

As the majority of the Fab is directed to the culture medium, this broth can be used for further Fab D1.3 purification work described in next chapter.

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3 Chromatographic purification of Fab D1.3

Abstract

Affinity and ion exchange chromatography techniques were used in order to purify Fab D1.3 from culture broth and/or osmotic shock fluid samples. In the first purification experiment, direct purification of Fab D1.3 on HiTrap Protein G column from a culture broth sample, having high conductivity of ~40 mS/cm, was not promising. HEWL was coupled with CNBr-Sepharose 4 Fast Flow matrix and employed for capture of the Fab from culture broth and osmotic shock fluid samples. By doing this chromatography, 21 to 27% of the existing Fab in the samples was captured on the Lysozyme column, and 48 to 55% Fab purity was yielded. Further purification from the Lysozyme column's eluates on HiTrap Protein G yielded 97% Fab D1.3 purity. The overall yield of the target Fab in the sequential Lysozyme affinity – Protein G affinity chromatography route was 18-33%. Cation Exchange chromatography, using HiTrap SP Sepharose XL, was also employed for capturing the Fab from a culture broth sample (pH 5.0, conductivity 5.0). Capture of the Fab from the culture broth sample was very efficient (>97%) by CEX chromatography, and 14% Fab purity was achieved in the eluates of the CEX column. Additional Fab purification from desalted eluates originated from the CEX column yielded >97% Fab D1.3 purity. The overall yield of the target Fab in the sequential CEX – Protein G affinity chromatography route was 83%, which was much higher than the figure (18-33%) achieved from the sequential Lysozyme affinity – Protein G affinity chromatography.

3.1 Introduction

Proteins are purified using purification techniques that separate according to differences in specific properties such as ligand biospecificity, charge, size and hydrophobicity. Affinity chromatography (AC) separates proteins on the basis of a reversible interaction between protein(s) and a particular ligand coupled to a chromatography matrix. Being highly selective and the only technique that enables the purification of a protein on the basis of its biological function or individual chemical structure, AC is ideal for a capture with purification levels in the order of several thousand-fold whenever an appropriate ligand is accessible for the protein of interest. The technique can be utilised to separate active proteins from the denatured or functionally different forms (Cuatrecasas and Anfinsen, 1971; Hage, 1991; www.chromatography.amershambiosciences.com). Electrostatic or hydrophobic interactions, van der Waals' forces and/or hydrogen binding can attach ligand and target protein. The interaction can be reversed to elute the target protein from the affinity medium by using a competitive ligand, or non-specifically, by altering the pH, ionic strength or polarity. Successful affinity purification requires a specific ligand that can be covalently attached to a chromatography matrix. The coupled ligand must retain its specific binding affinity for the target molecules and, after washing away unbound material, the binding between the ligand and target molecule must be reversible to allow the target molecules to be eluted in an active form. AC can be also used for removing specific contaminant, such as serine proteases, thrombin and Factor Xa. In typical affinity purification, affinity medium is equilibrated in binding buffer; sample is applied under conditions suitable for specific but reversible binding of target protein to a ligand; unbound materials are washed away, and by altering conditions favouring elution of the bound molecules, target protein is recovered in a purified concentrated form.

There are few bacterial receptors as ligands for antibodies and/or antibody fragments. These ligands have been employed in packed bed chromatography for purification of Immunoglobulins. Protein A, a 42 kDa cell surface protein of *Staphylococcus aureus*, is a ligand extensively used for Immunoglobulin purification. The primary binding site of Protein A on IgG is situated at the junction between the

C_H2 and C_H3 domains on the Fc fragment (Bak, 2004; Lindmark *et al*., 1981). Protein G, a ~60 kDa cell surface protein from group C and G *Streptococci*, can bind to IgG with high affinity. The Fc part of IgG molecule is mainly responsible for the interaction with Protein G, however, C_H1 domain of Fab also interact with Protein G (Björck and Kronvall, 1984; Derrick and Wigley, 1994). The native Protein G molecule also binds albumin; however, because serum albumin is a major contaminant of antibody sources, recombinant Protein G (rProtein G) lacking albumin domain has been developed and used for chromatographic purification of immunoglobulins. Protein L, a 76-106 kDa surface protein from *Peptostreptococcus magnu*, was also found to bind immunoglobulin through kappa light chain interaction, from which the name was suggested (Björck, 1988). Since no part of the heavy chain is involved in the binding interaction, Protein L binds a wider range of antibody classes, including IgG, IgM, IgA, IgE and IgD, and antibody fragments such as ScFv and Fab. However, Protein L binding restricted to those antibodies containing kappa chains. In humans and mice, most antibody molecules contain kappa light chains and the remainder have lambda light chains.

Ion exchange chromatography (IEC), which is based on the reversible interaction between charged solute molecules and oppositely charged moieties covalently linked to a chromatography matrix, is probably the most frequently used chromatographic technique for the separation and purification of proteins and other charged biomolecules (www.chromatography.amershambiosciences.com; Staby *et al*., 2000). It is feasible to have both positively and negatively charged exchangers. Positively charged exchangers have negatively charged counter-ions (anions) and are called anion exchangers (AEXs). Negatively charged exchangers have positively charged counter-ions (cations) and are termed cation exchangers (CEXs). The high capacity, the high resolving, the extensive applicability, the simplicity and controllability of the IEC are the reasons for the success for the method. Separation is obtained since different substances have different degrees of interaction with the ion exchanger due to differences in their charges, charge densities and distribution of charge on their surfaces. These interactions can be controlled by altering conditions such as ionic strength and pH. The differences in charge properties of biological compounds are often substantial, and as IEC is able to separate species with very minor differences

in properties, e.g. two proteins differing by only one charged amino acid, it is very powerful separation technique.

IEC can be used at all stages of a purification process (see Fig 3.1), i.e. during capture, intermediate purification, and polishing.

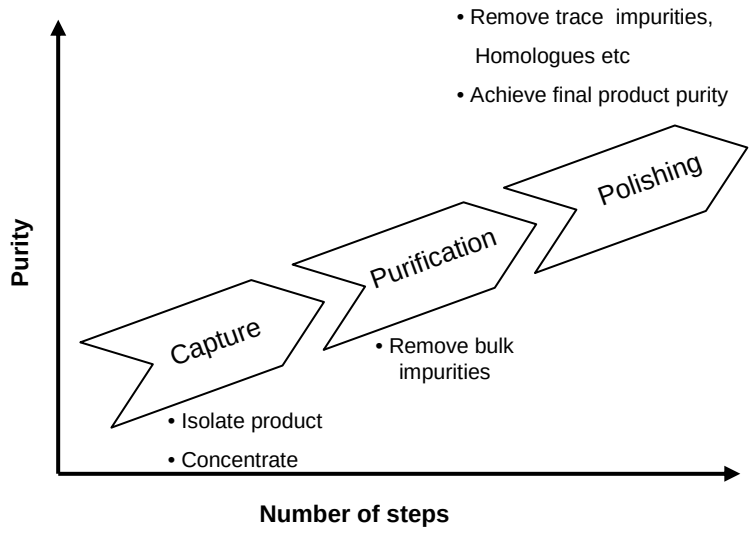

Fig. 3.1 Chromatography at various stages in downstream processing (Redrawn from Theodossiou, 2004).

In IEC, depending on the aim, one can select whether to bind the substances of interest and allow the contaminants to pass through the column, or to bind the contaminants and allow the substance of interest to pass through. The earlier method is generally more fruitful in the beginning of the purification process because it leads to a greater degree of fractionation and concentrates the substances of interest.

Ion exchange experiments are conducted in four main steps (Theodossiou, 2004). The first step is equilibration in which the ion exchanger is brought to a starting state, in terms of pH and ionic strength, which permits the binding of the desired solute molecules. The second step is sample application and association of the exchanger groups via exchangeable counter-ions, in which solute molecules bind reversibly to the matrix. The third step is removal of substances from the column by applying

elution conditions, which normally is increasing the ionic strength of the eluting buffer or changing its pH. By the introduction of an increasing salt concentration gradient, solute molecules are released from the column in the order of their strengths of binding, the most weakly bound substances being eluted first. The fourth step is the removal of substances not eluted from the column under the previous experimental conditions and re-equilibration at the starting conditions for another cycle of purification.

Ion-exchangers are applied in monoclonal antibody production. For example one-step purification of IgG and albumin from serum using coupled DEAE (TSK gel DEAE-5PW) and Protein G columns have been reported (Qi *et al*., 2001). IEC is also employed as polishing steps downstream of initial capture (e.g. on Protein A) due to their efficiency in the removal of contaminants such as DNA, viruses and endotoxin (Bak, 2004).

The aim of the work in this chapter was to highly purify Fab D1.3, which was needed for its structural studies in the presence of chemicals using high throughput circular dichroism (ht-CD) described in Chapter 4, from culture broth and/or osmotic shock fluid samples taken from fed-batch fermentation. In this work, purification of Fab D1.3 using various affinity and IEC chromatography supports, i.e. Hitrap Protein G, Lysozyme-activated CNBr-sepharose matrix/Hitrap Protein G, and Hitrap SP Sepharose XL/Hitrap Protein G, was investigated.

3.2 Materials and Methods

3.2.1 Materials

Chicken egg white Lysozyme (HEWL), sodium phosphate monobasic, sodium phosphate dibasic, Tris (hydroxymethyl)aminoethane hydrochloride, sodium bicarbonate, sodium chloride, sodium acetate, glycine, MES (2-(N-Morpholino)ethanesulfonic acid), MES sodium salt, diethylamine and dialysis tubing cellulose membrane were purchased from sigma (St. Louis, MO,USA). CNBr-

Sepharose 4FF matrix, HiTrap Protein G columns (1 mL), HiTrap SP XL columns (5 mL) and PD10 columns were purchased from GE Healthcare (Uppsala, Sweden).

3.2.2 Methods

3.2.2.1 Fab D1.3 purification

Purification from osmotic shock fluid and culture broth sample obtained from fed-batch fermentation, described in chapter 2, was carried out as below.

3.2.2.1.1 Fab D1.3 purification on HiTrap Protein G column

For this purpose, 1-mL HiTrap Protein G was equilibrated with 20 mM sodium phosphate buffer, pH 7.0. A 21 h culture broth sample (120 mL) from fed-batch cultivation (11 h post induction) was centrifuged at 9390 g for 600 s (Sigma 3K30, SciQuip, Shropshire, UK) and clarified by filtration through 0.45 µm syringe filters (Millipore, MA, USA). The sample (pH 7.0) was put through the HiTrap Protein G column using an AKTA explore system (GE Healthcare, Life Sciences, Buckinghamshire, UK) at a flow velocity of 1 mL/minute. After loading, the column was extensively washed with 20 mM sodium phosphate buffer, pH 7.0, and elution was made with 0.1 M glycine-HCl, pH 2.7 at the same flow rate. The eluate was instantaneously neutralized with 1 M Tris-HCl, pH 9.0, and stored at 4°C. The amount of Fab D1.3 in various fractions was measured using ELISA.

3.2.2.1.2 Fab D1.3 purification on HEWL-activated CNBr-Sepharose 4 Fast Flow matrix/ HiTrap Protein G column

<u>CNBr-Sepharose activation with HEWL</u>

Coupling HEWL to CNBr activated Sepharose was performed according to the manufacturer instruction. Briefly, 1 g CNBr-Sepharose 4FF matrix was washed with 15 medium volumes of cold (4°C) 1 mM HCl. Small wash portions (1 medium volume, 5 mL) was used and the mixture left 120 s for equilibration during each

washing step. The washed medium was then added to 5 mL solution containing 0.1 M $NaHCO_3$ (pH 8.3), 0.5 M NaCl and 10 mg HEWL for overnight shaking at 4°C. The reaction was followed by measuring UV-absorbance (at the wavelength 280 nm). In the next step, CNBr-Sepharose was washed with 5 medium volumes (5 × 5 mL) of coupling buffer to wash away HEWL. HEWL-coupled CNBr Sepharose was contacted with 0.1 M Tris-HCl, pH 8.0, for 2 h to block non-reacted groups on the medium. The coupled medium was eventually washed for 4 cycles using alternate low and high pH (0.1 M acetate buffer, pH 3-4, containing 0.5 M NaCl and 0.1 M Tris-HCl, pH 8.3, containing 0.5 M NaCl), in which 15 mL of each buffer applied and each buffer wash took 120 s.

The coupled medium stored in 0.1 M Tris-HCl (pH 8.3) containing 0.5 M NaCl at 4°C until required for further column packing. The medium in the column (Tricorn 10/50, 10 mm i.d.) was stored in 20% ethanol.

Purification

After developing HEWL-activated CNBr-Sepharose 4 Fast Flow matrix, the purification work was conducted. Purification from periplasmic fraction, i.e. osmotic shock fluid 1 described in chapter 2 (after 4 h post induction with 0.1 mM IPTG), and culture broth sample (after 11 h post induction) was performed on HEWL-activated CNBr-Sepharose 4FF column (5 mL). For this purpose, 480 mL culture broth and 250 mL OS_1 fluid were filtered through 0.45 µm cellulose acetate Millipore filters and loaded on the column at a flow velocity of 3.8 mL/minute in separate experiments. Two regimes of column washing, at the same flow rate (i.e. 3.8 mL/minute), were employed after loading; one was washing with 20 mM phosphate buffer, pH 7.0, and another one was washing with 20 mM phosphate buffer, pH 7.0, followed by 50 mM Tris-HCl buffer supplemented with 500 mM NaCl, pH 8.5. Elution was made with 50 mM diethylamine, pH 11.5. Eluted fractions containing Fab D1.3 from chromatography on the Lysozyme-Sepharose 4FF were pooled and desalted on PD10 columns prior to manual loading onto 1 mL HiTrap Protein G column pre-equilibrated with 20 mM sodium phosphate, pH 7.0. After extensive washing with the equilibration buffer, bound material was eluted with 0.1 M glycine-HCl, pH 2.7. The

eluate was neutralized immediately with 1 M Tris-HCl, pH 9.0, and stored at 4°C. Various fractions were analysed using SDS-PAGE, Western blotting and ELISA.

3.2.2.1.3 Fab D1.3 purification on HiTrap SP Sepharose XL/ HiTrap Protein G column

Purification from culture broth sample (after 11 hours post induction with 0.1 mM IPTG) was performed on a 5-mL HiTrap SP Sepharose XL column. Prior to application of cell-free culture fluid onto the column, the feedstock was extensively dialysed overnight against 15 mM sodium acetate, pH 5.0, at 4°C with changing the buffer in order to reduce both the conductivity (from ca. 40 to 5.0 mS/cm) and pH (from 7.0 to 5.0) for optimal sorption of the Fab on the cation exchange (CEX) matrix, The Dialysed culture broth sample was re-centrifuged (9390 g for 10 minutes) and filtered through 0.45 µm cellulose acetate Millipore filters. The sample (480 mL) was then loaded at a flow rate of 3.4 mL/minute on the HiTrap SP XL column pre-equilibrated with 15 mM sodium acetate buffer, pH 5.0. At the same flow velocity, the column was then washed with the equilibration buffer, and elution made with 20 mM MES (2-morpholinoethanesulfonic acid, 4.5 mM sodium MES, 1.5 M NaCl, pH 5.5). Eluted fractions containing Fab D1.3 from chromatography on HiTrap SP XL column were pooled and desalted on PD10 columns prior to manual loading onto a 1-mL HiTrap Protein G column pre-equilibrated with 20 mM sodium phosphate, pH 7.0. After extensive washing with the equilibration buffer, bound material was eluted with 0.1 M glycine-HCl, pH 2.7. The eluates were neutralized immediately with 1 M Tris-HCl, pH 9.0, and stored at -20 ^{o}C. Various fractions were analysed using SDS-PAGE, Western blotting and ELISA.

3.2.2.2 Analytical methods

SDS-poly-acryl amide gel electrophoresis (SDS-PAGE), Western blotting and ELISA were done as described in charter 2 (section 2.2.2.4). Mass spectrometry was performed by the Functional Genomics Laboratory at the University of Birmingham.

3.3. Results and Discussion

Various chromatography methods were used in order to capture and purify Fab D1.3 from the osmotic shock fluid and culture broth samples.

3.3.1 Fab D1.3 purification on HiTrap Protein G column

Though Protein G has strong affinity for the Fc regions of IgG molecule, it also shows weak affinity towards the C_H1 domain of Fab (Derrick and Wigley, 1994). Purification of various Fab molecules from Chinese hamster ovary (CHO) cell supernatant and the periplasmic fraction of *E. coli* on immobilised Protein G have been reported in literature (Humphreys *et al*., 1997; Kwack, 2000; and Proudfoot *et al*., 1992). Proudfoot *et al*. (1992) exploited this low-affinity interaction for the purification of chimeric mouse-human B72.3 Fab and $F(ab')_2$ fragments and showed that chimeric B72.3 Fab bound weakly to the Protein G-Sepharose so that it could be separated from F(ab')2 and eluted with a pH 7.0 wash, whereas B72.3 $F(ab')_2$ required elution at pH 2.0. In the present work, purification of Fab D1.3 using Protein G affinity chromatography was experimented. The chromatogram and ELISA figures relevant to purification of the Fab from culture broth directly on HiTrap Protein G column have been illustrated in Fig. 3.2. As it is evident from the ELISA data (Fig. 3.2), most (>99%) of the Fab applied to the column failed to bind, and diminutive quantities of the Fab were detected in the wash and elution fractions. This indicated that Protein G had very weak ability to attach the Fab under the experimental conditions (i.e. conductivity ~40 mS/cm, pH 7.0, sample volume 120 mL). The high conductivity (i.e. the high concentration of salt) in the culture broth might prevent the binding of Fab D1.3 to the Protein G column. In the works performed by other scientists on direct purification of various Fab fragments on Protein G columns, no detailed data (e.g. chromatography/conductivity values) had been given; therefore, it was impossible to compare the experimental conditions (e.g. conductivity) in this work with theirs.

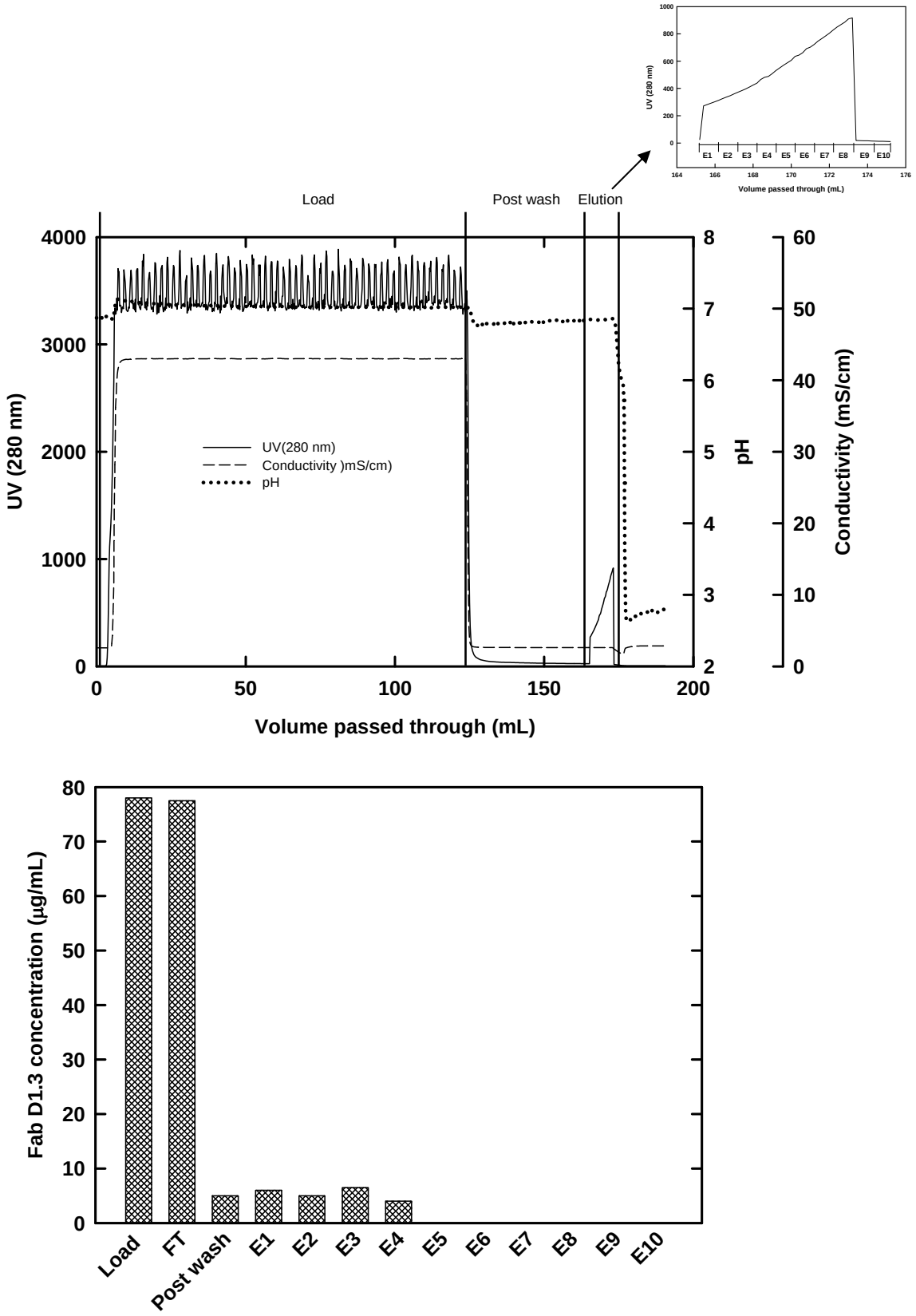

Fig. 3.2 Chromatogram (top) and ELISA data (bottom) for the purification of Fab D1.3 from a 21 h culture broth sample on a HiTrap Protein G column.

3.3.2 Fab D1.3 purification on HEWL-activated CNBr-Sepharose 4 Fast Flow matrix/ HiTrap Protein G column

Protein and other molecules containing primary amino groups can be coupled directly to the CNBr-activated Sepharose gel. Basic amino acids in HEWL, i.e. twelve arginine residues and six lysine residues, could react with cyanate ester groups in CNBr-activated Sepharose (Sasaki *et a*l., 2003). Purification of heavy and light chain variable domains (Fv) of the anti-Lysozyme D1.3 from culture broth and the periplasmic fraction of *E. coli* has been done on HEWL-Sepharose column (Cumber *et al*., 1992; Holmes *et al*., 1998; Winter *et al*., 2003). HEWL interacts extensively with V_K and V_H domains in Fab D1.3, forming three hydrogen bonds to the V_K domain and nine hydrogen bonds to the V_H domain (Ward *et al*., 1989). It has been shown that amongst various avian and human Lysozymes, D1.3 monoclonal antibody can specifically bind HEWL (Harper *et al*., 1987).

In the present work, purification of Fab D1.3 from culture broth and the periplasmic fraction (OS_1 fluid) on a Lysozyme-Sepharose affinity column was also tested. For purification of the Fab from culture broth on the Lysozyme-Sepharose column, the sample was loaded on the Lysozyme-Sepharose column and the column was then washed with 20 mM phosphate buffer, pH 7.0, and elution was made with 50 mM diethylamine, pH 11.5. The chromatogram, SDS-PAGE and Western blotting results relevant to this experiment have been shown in Fig. 3.3. Analysis of the elution fractions by SDS-PAGE confirmed the present of the Fab in the eluates, and showed that impurities accompanied with the Fab.

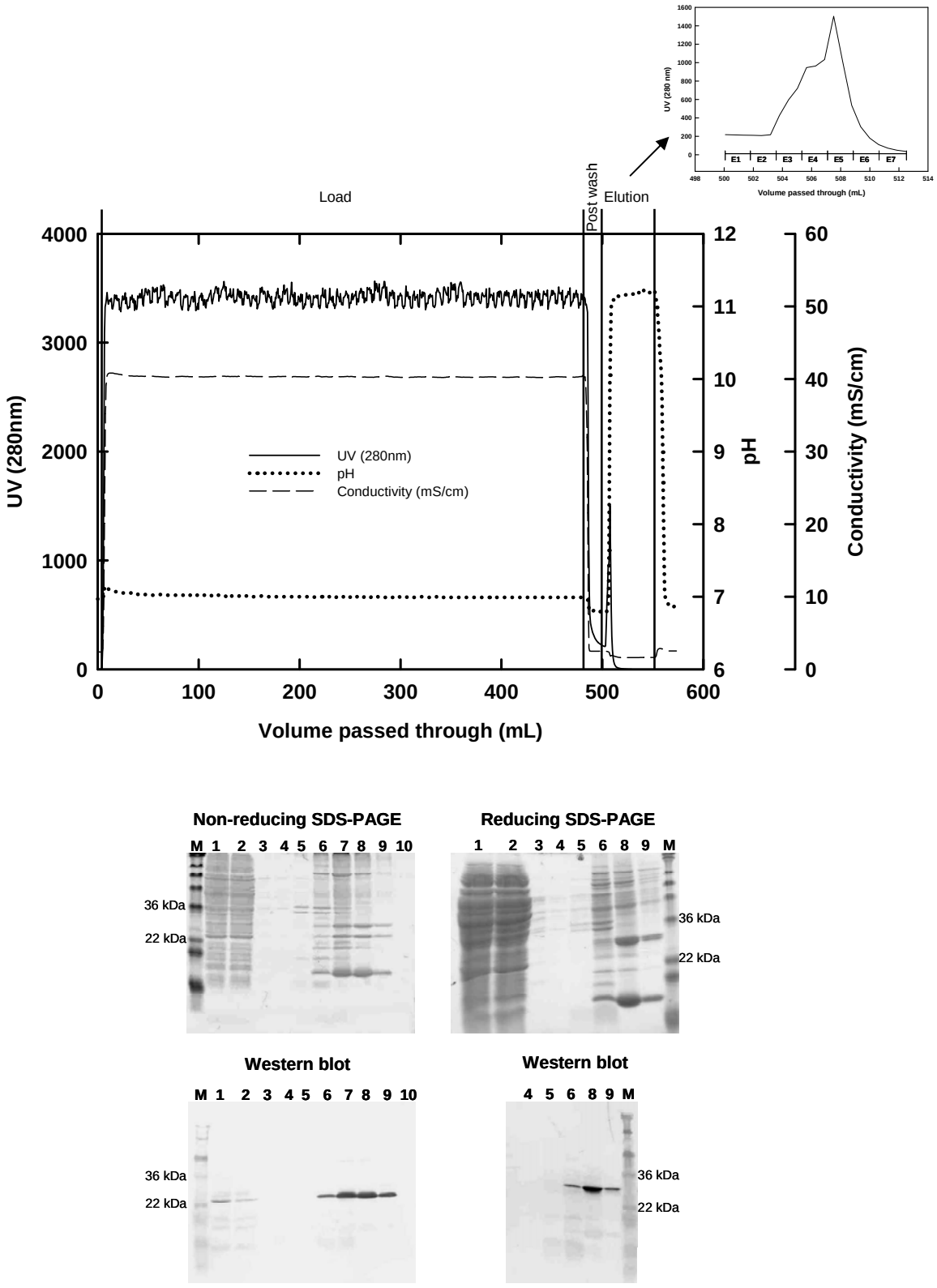

Fig. 3.3 Chromatogram (top), SDS-PAGE (middle) and Western blot (bottom) analyses for the purification of Fab D1.3 from culture broth (21 h culture) on a Lysozyme-activated Sepharose 4FF column. Phosphate buffer (20 mM, pH 7.0) was employed for equilibration and the post wash, and 50 mM diethylamine, pH 11.5 was used as the eluent. Lane: 1 – load; 2 – flow through; 3 – post wash; 4 to 10 – elution fractions; M – molecular weight markers.

For purification of Fab D1.3 from the periplasmic fraction (OS_1 fluid, 14 h culture) on the Lysozyme-Sepharose column , the post-load column washing regime was made with 20 mM phosphate buffer, pH 7.0, followed by 50 mM Tris-HCl buffer supplemented with 500 mM NaCl, pH 8.5, and elution was made with 50 mM diethylamine, pH 11.5. The chromatogram, SDS-PAGE and Western blotting results have been shown in Fig. 3.4. As evident from these results, additional protein was released during washing the column with 50 mM Tris-HCl buffer, pH 8.5, and no Fab D1.3 existed in the post-load wash fractions according to SDS-PAGE and Western blotting results. This indicates that an extra column wash with 50 mM Tris-HCl buffer, pH 8.5, prior to column elution step can unbind further impurities. Holmes *et al*. (1998) exploited this two-step post-load wash prior to elution of the Fv fragment of a humanized version of the mouse anti-Lysozyme D1.3 (HuLys) by 50 mM diethylamine from a Lysozyme-Sepharose affinity column. Western blot analysis also confirmed the present of the Fab in the eluates, but concomitant impurities were also observed (Figs 3.4). In the purification works done by other scientists using Lysozyme-Sepharose column, which discussed earlier in this section, no chromatogram or SDS-PAGE/Western blotting figures had been presented; therefore, the purity of the purified antibody fragment in those works was not manifest.

As the Fab in the elutions originated from the Lysozyme-Sepharose column was accompanied with impurities, even when the column was washed with the high salt buffer before the elution stage (see Figs 3.3 and 3.4), an extra step of purification on HiTrap Protein G was performed following desalting the eluates on PD10. By doing this highly pure Fab D1.3 was obtained, as indicated in SDS-PAGE and Western blotting results (Figs. 3.5 and 3.6). The top and bottom bands in lane 6 and the bottom band in lane 1 of non reducing SDS-PAGE (Fig 3.6) were analysed by mass spectroscopy. The mass spectroscopy data were analysed by Mascot search engine (http://www.matrixscience.com) to identify proteins from primary sequence database. Results (Tables 7.1 to 7.3 presented in the Appendix) demonstrated that the top and bottom bands in the lane 6 (corresponding to elution 1 of HiTrap Protein G column) were heavy and light chains of Fab D1.3, respectively, and the bottom band in lane 1 was not antibody fragment.

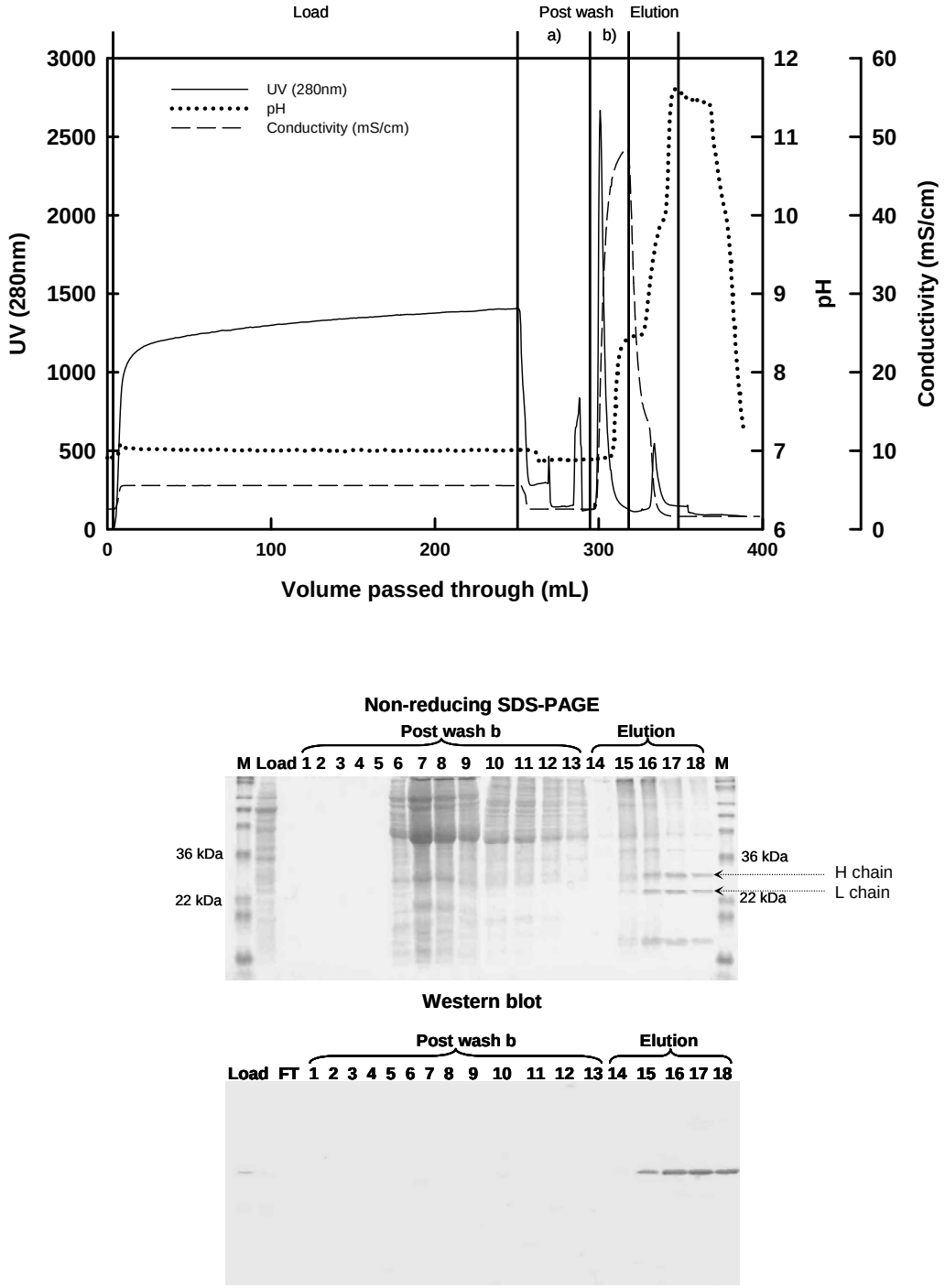

Fig. 3.4 Chromatogram (top), SDS-PAGE (middle) and Western blot (bottom) analyses for the purification of Fab D1.3 from OS1 (14 h culture) on a Lysozyme-activated Sepharose 4FF column. Phosphate buffer (20 mM, pH 7) was employed for equilibration and post wash a. The 2nd wash (post wash b) was performed with a high salt buffer (50 mM Tris-HCl buffer supplemented with 500 mM NaCl, pH 8.5) and elution was achieved with 50 mM diethylamine, pH 11.5. The molecular weight of each chain of Fab D1.3 is about 25 kDa.

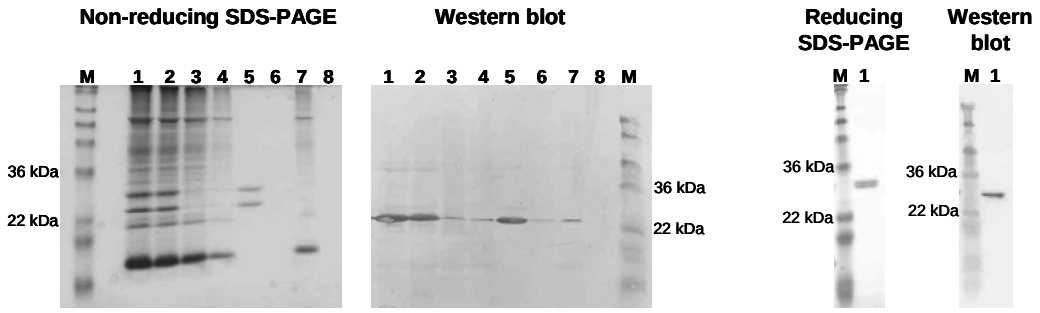

Fig. 3.5 SDS-PAGE gels and corresponding Western blots of selected fractions from the purification of partially purified Fab D1.3 on HiTrap Protein G. Eluted fraction 7 from chromatography on Lysozyme Sepharose 4FF (Fig. 3.3) was desalted on a PD10 column prior to loading onto a HiTrap Protein G column. For the non-reducing SDS-PAGE gel (far left) and corresponding Western blot (second from the left) a common lane designation applies, i.e.: M – molecular weight markers; 1 – pre-load elution pool (Fig. 3.3) prior to PD10 desalting; 2 – PD10 desalted load; 3 – flowthrough; 4 – post load wash; 5 to 8 – elution fractions. For the reducing SDS-PAGE gel (second from the right) and corresponding Western blot (far right) lane M denotes molecular weight markers and lane 1 is elution fraction 1.

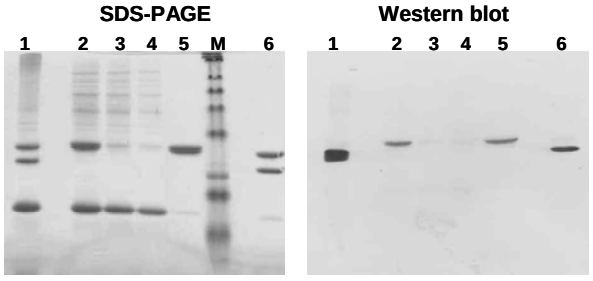

Fig. 3.6 SDS-PAGE gel (left) and Western blot (right) analyses following purification of partially purified Fab D1.3 on HiTrap protein G. Pooled eluted fractions (15-18) from chromatography on Lysozyme Sepharose 4FF (Fig. 3.4) were desalted on PD10 columns prior to loading onto a Hitrap Protein G column. Lane: 1 – PD10 desalted load (non-reduced); 2 – PD10 desalted load (reduced); 3 – flowthrough (reduced); 4 – post wash (reduced); 5 – eluate 1 (reduced); 6 – eluate 1 (non-reduced); M – molecular weight markers.

The comparison of isolated proteins on reducing and non-reducing SDS-PAGE provides an easy way to determine if a protein of interest might be a multimeric complex (Corely, 2004). By doing non-reducing SDS-PAGE, it was revealed that the heavy and light chains of the Fab molecule were not associated covalently inasmuch as two distinctive close bands observed under non-reducing condition. It is also noticeable that in all SDS-PAGE analyses done on the fractions containing Fab, the locations of the heavy and light chains on the gel were different under reducing and non-reducing conditions. Under reducing conditions, heavy and light bands (with very near molecular weight) on the gel were overlapped, most likely, due to a more extended structure. In reducing (denaturing) SDS-PAGE, the presence of beta-mercaptoethanol serves two functions. First, it reduces the covalent bonds that might exist between multimeric protein complexes. Second, it reduces covalent bonds that exist within a protein so that it achieves a more extended structure when boiled in SDS, which improves resolution and permits more accurate estimates of its molecular weight mass (Corley, 2004). Thus, under non-reducing conditions, proteins can migrate aberrantly on SDS-PAGE gels.

Tables 3.1 and 3.2 illustrate the chromatography data relevant to the Fab purification from culture broth and OS_1 fluid, respectively, by a sequential Lysozyme affinity – desalting Gel filtration (GF) – Protein G affinity chromatography route. It is evident from these data that the purification by Lsozyme-Sepharose column yielded 48% and 55% Fab purities for purification from culture broth sample and osmotic shock fluid, respectively. The Fab eluted from the Protein G column was substantially purified, i.e. reaching>97%, but the overall yields of the target Fab were rather low (i.e. 3.5 and 4 mg; or 18% and 33% for purification from the 10 h post induction culture broth and 4 h post induction osmotic shock fluid respectively).

Table 3.1 Chromatography data for purification of Fab D1.3 from culture broth (21 h culture) on Lysozyme-activated Sepharose 4FF/ HiTrap protein G column.

Step		Volume (mL)	D1.3 (mg)	Total protein (mg)	Target yield (%)	Total protein yield (%)	Purity (%)	PF
Lysozyme-activated sepharose (5-mL)	Load	480.00	19.58	1888.20	100.00	100.00	1.04	1.00
	FT	480.00	15.55	1864.80	79.42	98.76	0.83	
	Wash	20.00	0.49	16.53	2.50	0.88	2.96	
	Elution	*12.00*	*3.43*	*7.03*	*17.52*	*0.37*	*48.79*	*47.05*
HiTrap Pr. G column (1mL)	Load	3.50	3.43	7.00	17.52	0.37	49.00	
	FT	3.50	0.04	0.21	0.20	0.01	19.05	
	Wash	5.00	0.01	0.02	0.03	0.00	25.00	
	Elution	*4.00*	*3.40*	*3.50*	*17.36*	*0.19*	*97.14*	*93.68*

Table 3.2 Chromatography data for purification of Fab D1.3 from OS1 fluid (14 h culture) on Lysozyme-activated Sepharose 4FF/ HiTrap protein G column.

Step		Volume (mL)	D1.3 (mg)	Total protein (mg)	Target yield (%)	Total protein yield (%)	Purity (%)	PF
Lysozyme-activated sepharose (5-mL)	Load	250.00	12.23	228.20	100.00	100.00	5.36	1.00
	FT	250.00	7.80	202.50	63.78	88.74	3.85	
	Wash	56.00	0.20	18.30	1.64	8.02	1.09	
	Elution	*12.00*	*4.14*	*7.43*	*33.85*	*3.26*	*55.72*	*10.40*
HiTrap Pr. G column (1mL)	Load	2.50	4.14	7.42	33.85	3.25	55.80	
	FT	2.50	0.04	2.22	0.33	0.97	1.80	
	Wash	5.00	0.07	1.34	0.57	0.59	5.22	
	Elution	*8.00*	*4.00*	*4.05*	*32.71*	*1.77*	*98.77*	*18.43*

3.3.3 Fab D1.3 purification on HiTrap SP Sepharose XL/ HiTrap Protein G column

Cation exchange (CEX) chromatography can be used as a very helpful technique for concentrating proteins of interest in the beginning of a purification process. In the present work, capture of Fab D1.3 from culture broth sample by cation exchange chromatography (5-mL HiTrap SP Sepharose XL column) was also investigated. For optimal sorption of the Fab on the cation exchange matrix, the pH and conductivity of the load were adjusted to 5.0 and 5.0 mS/cm, respectively. The conditions required for this purification experiment were obtained from Ljunglöf *et al's* work on the ion exchange chromatography of antibody fragment (Fab) (Ljunglöf *et al*., 2007), in which they stated a dynamic binding capacity (DBC at 10% breakthrough) of ca. 120 mg

Fab/mL adsorbent at pH 5.0 and conductivity 5.0 mS/cm. The binding, washing and eluting buffers were also chosen according to Ljunglöf *et al's* work. By doing SDS-PAGE and Western blot analyses, the presence of Fab with concomitant protein impurities in the elution fractions of the SP Sepharose column was confirmed (Fig. 3.7).

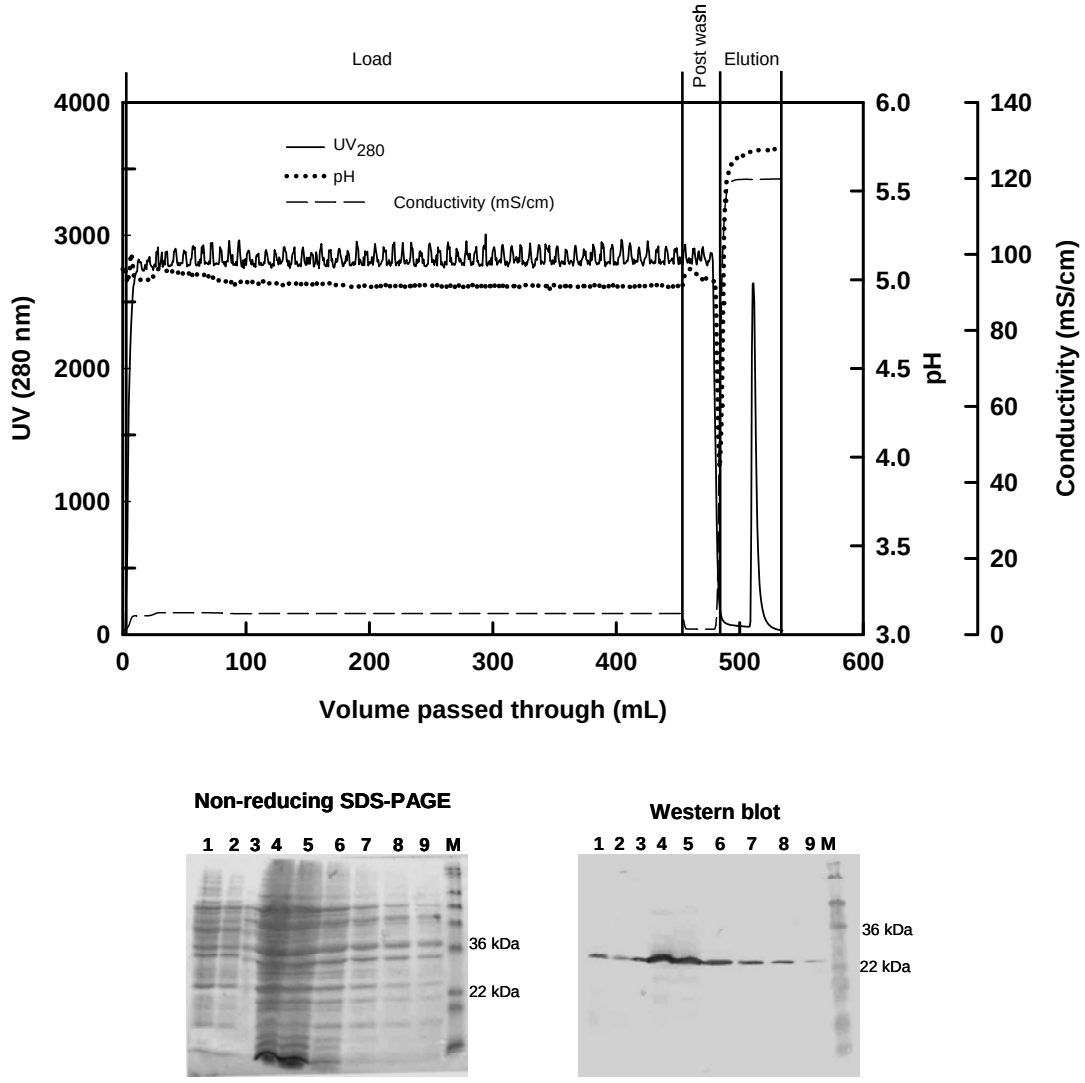

Fig. 3.7 Chromatogram (Top), non-reducing SDS-PAGE (bottom left) and corresponding Western blot (bottom right) analyses for the purification of Fab D1.3 from culture broth (21 h culture) on a HiTrap SP Sepharose XL column. Sodium acetate (15 mM, pH 5.0) was employed for equilibration and the post wash. Elution was achieved by 20 mM MES, 4.5 mM sodium MES, 1.5 M NaCl (pH 5.5). Lane: 1 – load; 2 – post wash; 3 to 9 – elution fractions; M – molecular weight markers.

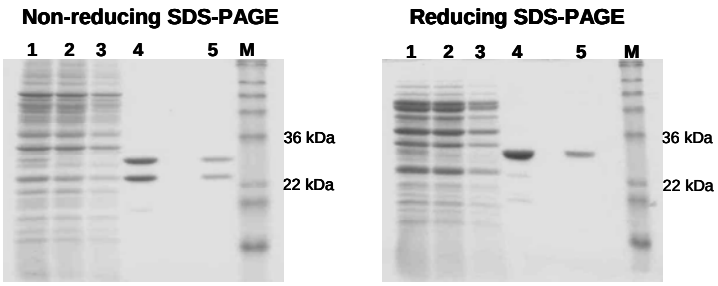

Fig. 3.8 Non-reducing SDS-PAGE (left) and reducing SDS-PAGE (right) gels following purification of partially purified Fab D1.3 on HiTrap protein G. pooled fractions (3-9) from chromatography on HiTrap SP Sepharose XL column (Fig. 3.7) were desalted on PD10 columns prior to loading into a HiTrap protein G column. Lane: 1 – PD10 desalted load; 2 – flowtrough; 3 – post wash; 4 and 5 – eluate 1 and 2, respectively; M – molecular weight markers.

To obtain high pure Fab D1.3, a further step of purification on HiTrap Protein G was performed using eluates desalted on PD10 column. Highly pure Fab D1.3 was obtained as illustrated in the SDS-PAGE and Western blotting results (Fig. 3.8).

Table 3.3 shows the chromatography data related to the Fab purification from culture broth sample by a sequential SP Sepharose XL cation exchange (CEX) – desalting Gel filtration (GF) – Protein G affinity chromatography route. When compared to the quantity of the Fab measured before dialysis, approximately 60 per cent loss of the Fab occurred during overnight dialysis of culture broth sample, done so as to reduce conductivity and pH (i.e. the Fab titration was dropped from 40.8 mg/L to 17.3 mg/L). The solution obtained from dialysis was cloudy and the precipitates were visible on the bottom of tubes after clarification with centrifugation. As it is shown in Table 3.3, the Fab capture from the culture broth sample on the cation exchanger was very successful (> 97% capture), and the pooled eluate from this CEX step yielded a ca. 14% Fab purity and a 22.5-fold purification factor. Further Fab purification from desalted CEX eluates on Protein G column resulted in a highly pure Fab (with >97% purity). The overall yield and purification factor in this ‘CEX–GF–Protein G affinity’ route were 83% and 153-fold, respectively, being significantly higher than that

obtained previously using the 'Lysozyme affinity–GF–Protein G affinity' procedure (see Tables 3.1 and 3.2).

Table 3.3 Chromatography data for purification of Fab D1.3 from culture broth (21 h culture) on HiTrap SP Sepharose XL/ HiTrap protein G column.

Step		Volume (mL)	D1.3 (mg)	Total protein (mg)	Target yield (%)	Total protein yield (%)	Purity (%)	PF
SPXL column (5 mL)	Load	480.0	8.3	1303.4	100.0	100.0	0.6	1.0
	FT	480.0	0.2	1235.7	1.8	94.8	0.0	
	Wash	25.0	0.9	17.5	10.2	1.3	4.9	
	Elution	*30.0*	*7.2*	*50.2*	*86.7*	*3.9*	*14.3*	*22.5*
HiTrap Pr. G column (1mL)	Load	7.0	7.2	50.2	86.7	3.9	14.3	
	FT	7.0	0.2	35.0	2.8	2.7	0.7	
	Wash	5.0	0.0	0.1	0.6	0.0	46.0	
	Elution	*2.5*	*6.9*	*7.0*	*82.5*	*0.5*	*97.9*	*153.7*

3.4 Conclusions

Streptococcal Protein G can bind strongly to the Fc region (between C_H2 and C_H3 domains), and weakly to C_H1 domain. Therefore, Protein G can be used for purification of whole IgG molecules, and some reports have shown that it is a potential ligand for purification of antibody fragments as well (Björck and Kronvall, 1984; Derrick and Wigley, 1994). In this work purification of Fab D1.3 by various chromatography routes has been investigated. Fab D1.3 binds very weakly (<1%) to Protein G column, when the culture broth sample (with conductivity ~40 mS/cm and pH 7.0) directly is loaded on the Protein G column.

Amongst avian and human Lysozymes, D1.3 monoclonal antibody can specifically bind HEWL (Harper *et al*., 1987). In this work, it has been demonstrated that Fab D1.3 can be purified from 480 and 250 mL culture broth and osmotic shock samples, respectively, on a 5-mL HEWL column with 48 to 55% purity. The Fab with such purity can bind (>97%) to protein G when the pooled HEWL column eluate is desalted on PD10 column using 20 mM sodium phosphate, pH 7.0. As a result, Fab D1.3 can be purified >97% using coupled HEWL and Protein G affinity columns.

However the overall yield of such chromatography step is low (18-33%) due to low binding capacity of the Lysozyme column.

Cation exchangers have a very good capacity for capturing and concentrating target proteins in the early stage of purification process. HiTrap SP Sepharose XL has been shown a high binding capacity for Fab (120 mgFab/mL adsorbant) at pH 5.0 and conductivity 5.0 (Ljunglöf *et al*., 2007). HiTrap SP Sepharose XL binds and captures Fab D1.3 (>97%) from 480 mL culture broth (with pH 5.0 and conductivity 5.0 mS/cm), and as a result concentrated target protein (with 14% purity) is achieved. The Fab with such purity can bind greatly to protein G when the pooled CEX column eluate is desalted on PD10 column using 20 mM sodium phosphate, pH 7.0. Therefore, Fab D1.3 can be purified >97% using coupled HiTrap SP Sepharose XL and Protein G columns. The overall yield of coupled HiTrap SP Sepharose XL and Protein G columns is considerably high (83%), and therefore appears more suitable than coupled HEWL and Protein G affinity columns for purification of Fab D1.3.

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4 The effect of periplasmic release reagents on the secondary structure and biological activity of three periplasmically expressed recombinant proteins

Abstract

High throughput circular dichroism (ht-CD) system was employed to evaluate the structural alterations occurred in bata-lactamase, alpha-amylase, and Fab D1.3 in the presence of different chemicals. In the first ht-CD experiments, the suitable protein concentration (1 mg/mL) required for such spectroscopic system was determined by measuring the intensity of CD spectra recorded for various concentrations (0.2-2 mg/mL) of bovine serum albumin (BSA). The reproducibility of the CD system was confirmed by recording the CD spectra of 1mg/mL BSA run consecutively into the system. The CD spectra of various chemicals were recorded, and chemicals which did not increase high tension (HT) voltage above 600 V within far-UV region were chosen for further ht-CD investigations. The CD spectra of 1mg/mL beta-lactamase, alpha-amylase, and Fab D1.3 were recorded in the presence of various chemicals (surfactants, chelating agents, chaotropic agents, and solvents), and the extent of changes in the secondary structure of proteins was evaluated in far-UV region. The changes in the CD spectra increased with increasing the concentration of chemicals. The biological activity of beta-lactamase and alpha-amylase, and the binding capability of Fab D1.3 to hen egg white HEWL immobilised on microplate were measured in the presence of various chemicals. By comparing CD results with biological activity results, it was divulged that certain chemicals might induce alterations in the secondary structure of proteins without reducing the biological activity of proteins, and vice-versa.

4.1 Introduction

The periplasm of *E. coli* has been recognized as a very suitable place for expression of recombinant proteins, especially for those having disulphide-bonds in their structures. The oxidizing environment of the periplasm, the periplasmic disulphide bond-forming (Dsb) proteins, isomerases, and chaperones assist the formation of properly folded target protein (Berkmen e*t al.*, 2007; Betton, 2007; Derman and Beckwith, 1991; Richarme and Caldas, 1997). In the beginning of a downstream process for the extraction and purification of periplasmically-expressed proteins, the selective recovery of periplasmically located proteins is of a great interest as this will reduce the number, and as a result the cost, of downstream operation units. There are many reagents that might be used for targeting and permeabilising the outer membrane of *E. coli* and other gram-negative bacteria (Nikaido and Vaara, 1985; Vaara, 1992). These reagents might have adverse impacts on the structure and biological activity of periplasmically-located proteins; thus, the influence of such reagents on the structure and biological activity of target proteins should be studied prior to applying them in cell permeabilisation. 'Safe' reagents can be then utilized in the outer membrane permeabilisation studies.

Circular dichroism (CD), which is a spectroscopic method based on the differential absorption of left- and right-circularly polarized light by optically active molecules, is a standard technique for analysis of secondary structure of biopolymers such as proteins. CD offers a myriad of advantages over X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy (Manavalan and Johnson, 1985; Miles and Wallace, 2006), and is used extensively for structural studies. (Johnson, 1988 and 1990; Kelly et al., 2005; Wallace et al., 2003; Wallace and Janes, 2001; Whitmore and Wallace, 2007). CD is a rapid method requiring small amounts of sample; it shows a high sensitivity to diminutive changes in secondary structure; it does not entail sample preparations (such as crystallization), high concentrations and extreme conditions; it enables users to analyse biomolecules in solutions and can be utilized to explore alterations in secondary structure in a broad range of solvent composition, experimental solution conditions and temperature.

In this chapter we have studied the use of a newly developed high throughput circular dichroism (ht-CD) system to rapidly identify safe conditions for chemically-mediated release of target proteins from the periplasm of *E. coli*. The effects of various concentrations of a large number of recognised cell permeabilisation reagents on the secondary structure of beta-lactamase, alpha-amylase and Fab D1.3 were first screened by ht-CD. The impact of such chemicals on the biological activity of these proteins was then studied, and any correlations between the CD results and activity assays determined.

4.2 Materials and Methods

4.2.1 Materials

Sodium deoxycholate (DOC), cetyltrimethylammonium bromide (CTAB), benzalkonium chloride (BAC), Triton X-100, Tween 20, Brij-35, ethylenediaminetetraacetic acid (EDTA), diethanolamine (DEA), nitriolotriacetic acid (NTA), sodium hexametaphosphate (SHMP), urea, guanidinium chloride (GndCl), diethyl ether, pyridine, isoamyl alcohol, (hydroxymethyl)aminomethane (Tris), sodium acetate, sodium phosphate monobasic, sodium phosphate dibasic, bovine serum albumin (BSA), sodium salt of benzylpenicillin, alpha-amylase from *Aspergillus orizae* and beta-lactamase from *Enterobacter cloacae* were obtained from Sigma-Aldrich (Gillingham, UK). Sodium dodecyl sulphate (SDS) was supplied by Bio-Rad Laboratories (Hercules, CA, USA). Hexane, xylene, chloroform, ethanol, acetone, glacial acetic acid and potassium iodide were obtained from Fisher Scientific (Loughborough, UK). Benzene, toluene and iodine were supplied by BDH Laboratory supplies (Poole, UK). Fab D1.3 was from purification from culture broth on HiTrap SP Sepharose XL/ HiTrap protein G HP column, as previously described in detail in Chapter 3.

4.2.2 Methods

4.2.2.1 High throughput method (ht-CD)

4.2.2.1.1 Quartz flow cell sampling analysis

Various concentrations of BSA (0.2-2 mg/mL) were prepared in double distilled water (DDH_2O). A 96-well microplate was loaded in a repeating pattern of protein and control (DDH20), each well containing 200 µl. Plate was placed within the intelligent autosampler, and a sampling program initiated. Fifty microlitres of the solution in each microplate well was sampled by the autosampler and injected by HPLC pump into the quartz flow cell having 2 mm path length. Once the flow in the cell was stopped, CD spectrum was taken. The spectrum was measured using parameters that allowed for a rapid accumulation of data (three scans were taken at 100 nm/minute between 270 and 190 nm with a 1 nm bandwidth and averaged). Data was viewed using the Spectra Management for Windows program during analysis and automatically saved by the program Spectra.

4.2.2.1.2 Ht-CD method reproducibility

A sample of 1 mg/mL BSA was prepared in 25 mM phosphate buffer pH 7.0, distributed in several wells on microplate in a repeated pattern of protein solution and control (25 mM phosphate buffer pH 7.0), and analysed in the high throughput system as mentioned above.

4.2.2.1.3 Ht-CD for combination of chemicals and proteins

The CD spectra of chemicals alone and mixtures of chemicals and proteins were measured. For this purpose, a 96-well autosampler was used to deliver the samples to the CD spectrometer. Each well of the sample plate was loaded with 100 µL of double concentrated chemicals and 100 µL of double concentrated pure proteins (2 mg/mL made in 50 mM phosphate buffer pH 7.0). For each chemical and protein tested, two wells containing 200 µL were produced; one containing desired protein in

the buffer and chemical, and the other containing the buffer and chemical, but no protein. This second sample provided a baseline CD spectrum which was taken away from the spectrum containing the protein to yield the true protein CD spectrum. Plates were placed within the intelligent autosampler, and a sampling program initiated. Fifty microlitres of the solution in each microplate well was sampled by the autosampler and injected by HPLC pump into a quartz flow cell having 2 mm path length. Once the flow in the cell was stopped, CD spectrum was taken. The spectrum was measured using parameters that allowed for a rapid accumulation of data (three scans were taken at 100 nm/minute between 250 and 190 nm with a 1 nm bandwidth and averaged). Data was viewed using the Spectra Management for Windows program during analysis and automatically saved by the program Spectra.

After CD measurements were made, the remaining solutions in the microplate were utilized in order to assess the retention enzyme activity of the proteins in the presence of various chemicals as mentioned below.

4.2.2.2 Analytical Ultra Centrifugation (AUC) studies

Double concentrated proteins and chemicals were mixed together to have appropriate final concentrations (ca. 0.5 mg of alpha-amylase, beta-lactamase, or Fab D1.3 per mL chemicals). Sedimentation velocity experiments were conducted in a ProteomeLab XL-1 Analytical Ultracentrifuge (Beckman Coulter Inc., Fullerton, CA, USA). The experiments were performed overnight at 100,000 g and 20°C. The velocity data were analysed using SEDFIT (Schuck, 2000).

4.2.2.3 Beta-lactamase assay

Beta-lactamase activity was assayed using a microtitre plate, according to a method adapted from Sargent (1968), in which penicilloic acid, the product of reaction between beta-lactamase and penicillin G, makes the iodine colourless, and consequently, the decrease in absorbance due to iodine can be used as estimation for enzyme activity. The following solutions were made freshly prior to the start of the assay: 4 mg of sodium salt of benzylpenicillin per mL of 50 mM sodium phosphate

buffer, pH 7.0; stock iodine solution containing 20.3 g I_2 and 100 g KI in 500 mL of distilled water; iodine reagent, prepared by adding 1.25 mL of stock iodine solution to 98.25 mL of sodium acetate buffer, pH 4.0. Routinely, 100 µL of sample were added to 20 µL of sodium salt of benzylpenicillin solution and following incubation at 37°C, for 180 s, the reaction was stopped by adding 200 µL of iodine reagent. The absorbance of the solution was then read immediately at 450 nm using a Promega microplate reader. As chemicals might change the colour intensity of iodine reagent in the beta-lactamase activity assay, for each chemical an individual blank containing 0.1 mL of chemical/buffer, 20 µL of 50 mM Na-phosphate buffer pH 7.0 and 200 µL iodine reagent was prepared.

4.2.2.4 Alpha-amylase assay

Alpha-amylase activity was measured using a modified microtitre plate assay based on the method of Blanchin-Roland and Masson (Blanchin-Roland and Masson, 1989), in which activity is determined by measuring the rate of decrease in absorbance of a coloured starch/I_2 complex. For this purpose, soluble starch 0.5% (w/v) was made in 15 mM sodium phosphate buffered pH 5.8, previously heated to boiling point and filtered through No. 1 grade Whatman filter paper (Whatman International Ltd, Maidstone, UK) whilst hot. The iodine reagent was prepared fresh by diluting 0.2 mL of stock solution (2.2% (w/v) iodine and 4.4% (w/v) KI) into 100 mL of 2% (w/v) potassium iodine solution. Routinely, 150 µL of the preincubated starch solution was added to an equal volume of the preincubated sample and the mixture was incubated at 50°C for 180 s (Infors AG type AK120 microplate incubator shaker, Infors HT, Bottmingen, Switzerland). Thereafter, 15 µL of the reaction mixture was pipetted into 300 µL of the iodine reagent and the absorbance of the solution was measured at the wavelength of 600 nm using a Promega microplate reader (Promega Glomax-Multi detection system, Turner Biosystems Inc., Sunnyvale, CA, USA). As chemicals might change the colour intensity of starch/I_2 complex in the alpha amylase activity assay, for each chemical an individual blank containing 150 µL chemical/buffer and 150 µL starch solution was prepared.

4.2.2.5 Sandwich ELISA method for Fab D1.3 quantification

ELISA, without diluting down the samples, was performed according to the procedure described in Chapter 2, section 2.2.2.4.2.

As the enzymatic quantifications methods and the sandwich ELISA technique are sensitive to the concentration of proteins, and because of this serial dilutions are usually made in a buffer. However in this work no dilution was made as there was the possibility of removing (diluting) the additions and as a result protein refolding. Therefore, due to lack of dilution process, the high sensitivity of these biological activity methods may be questionable and as a result minute reduction in the biological activity may not be detected. However, where the biological activity of the proteins is severely reduced, the high sensitivity of the assays is not debatable.

4.3 Results and Discussion

4.3.1 Checking the accuracy of the ht-CD system by using BSA

CD is an excellent method for analysing the secondary structure of proteins and peptides in solution, and valuable estimates of protein conformation can be acquired from data obtained only between 240 and 200 nm (Greenfield, 1996). In the present work, a newly developed ht-CD system – composing of a spectropolarimeter having the quartz flow cell with 2 mm path length, an autosampler, and an HPLC pump injector – was employed to study the structural changes of periplasmically-expressed proteins in the presence of various chemicals. As the CD spectrum of a protein must be adequately intense for interpreting the data and the intensity of a CD spectrum is relied directly on the protein concentration; therefore, the CD spectra of different concentrations (0.2-2 mg/mL) BSA made in 25 mM phosphate buffer pH 7.0, were used for testing the system. Figure 4.1 shows the spectra collected from this experiment, and it is evident that the intensity of CD spectra was dependent on the concentration of BSA and this correlation was very significant (Fig. 4.1 c). The concentration of 1 mg protein/mL solution gave rise to sufficiently strong CD spectrum, and was used for further ht-CD investigations.

To check the reproducibility of the ht-CD system, 1 mg/mL BSA in 25 mM phosphate buffer (pH 7.0) was sampled seven consecutive times by the autosampler, and after each sampling the spectra were recorded. As illustrated in Fig. 4.2, the spectra remarkably overlaid each other, and this proved the reproducibility of such system.

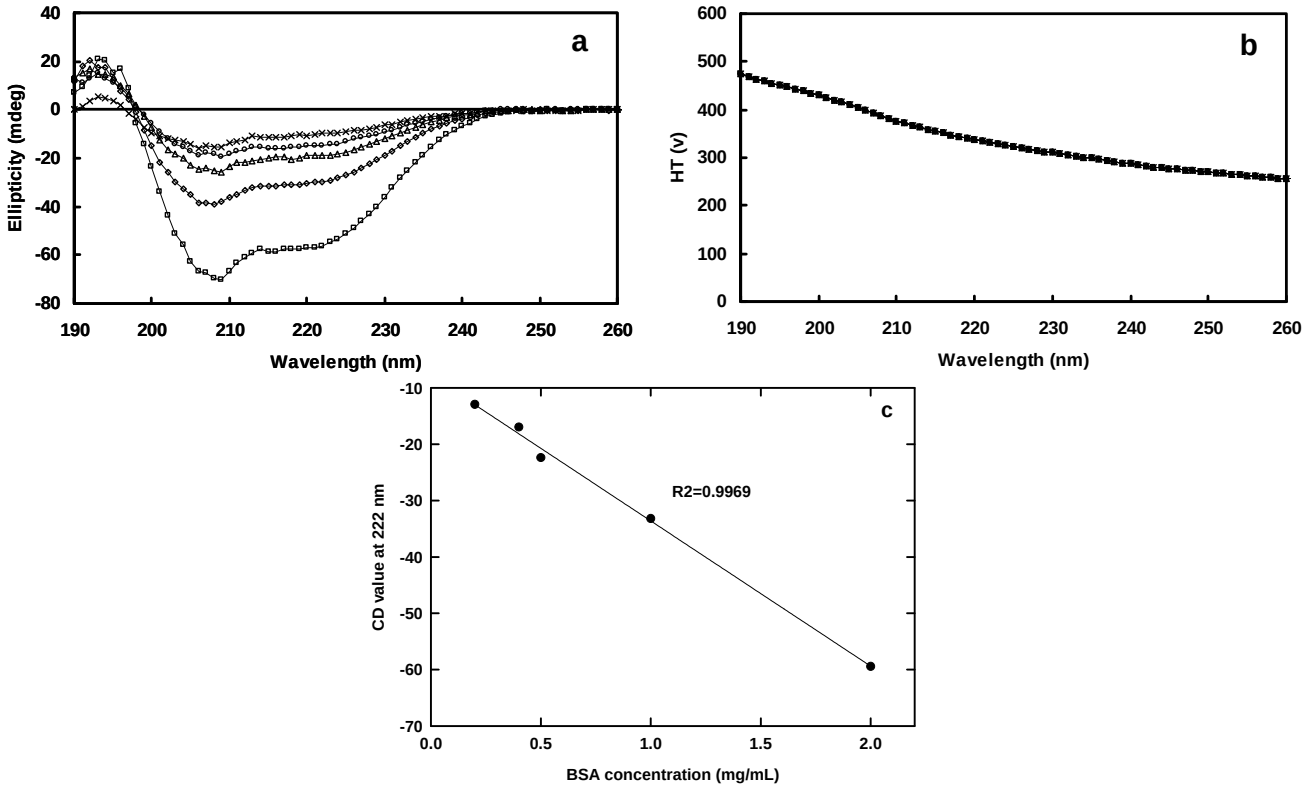

Fig. 4.1 CD spectra (a), the corresponding HT signals (b), and correlation between CD signal at 222 nm and BSA concentration (c). Spectra were recorded in a 2 mm path length capillary cell on a Jasco J-810 spectropolarimeter at a scan speed of 100 nm/minute, response 2 s, and a bandwidth of 1 nm. Three scans were accumulated for each sample. Key: BSA concentration made in 25 mM phosphate buffer pH 7.0 (mg/mL): (—×—) 0.2; (—○—) 0.4; (—△—) 0.5; (—◇—) 1.0; (—□—) 2.0.

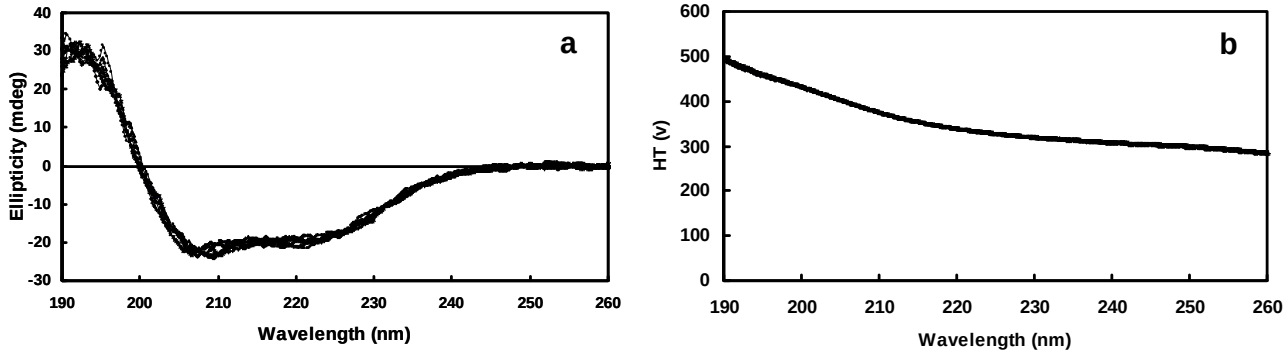

Fig. 4.2 CD spectra (a), the corresponding HT signals (b) of 1 mg/mL BSA sampled seven consecutive times in the ht-CD system. Spectra were recorded in a 2 mm path length capillary cell on a Jasco J-810 spectropolarimeter at a scan speed of 100 nm/minute, response 2 s, and a bandwidth of 1 nm. Three scans were accumulated for each sample.

4.3.2 Ht-CD studies of beta-lactamase, alpha-amylase, and Fab D1.3 in the presence of various chemicals

The function of a protein is dependent on its three dimensional structure. Change in pH can modify the structure of proteins by altering electrostatic interactions between charged amino acids. Alteration in pH may also change the charge of the substrates and as a result affect their binding to the active site of the enzyme (Nagamine *et al*., 1987).

Histidine (His), glutamic acid (Glu) and aspartic acid (Asp) located in the active site cleft of *Aspergillus oryzae* alpha-amylase participate in substrate binding and catalytical activity (Matsuura *et al.*, 1984; Toda *et al.*, 1982). It has been reported that this enzyme is stable in the pH range of 4.5 to 9, and is inactivated rapidly at pH values below 4 and above 9 (Ebisu *et al.*, 1993).

Glutamic acid (Glu) and serine in the active site of beta lactamases are involved in the catalytical pathway of these enzymes and mutations of Glu results in enzymes exhibiting significantly decreased acylation and deacylation activity (Damblon *et al*., 1996). The optimum pH for beta-lactamase (from Enterobacter cloacae) activity is 7-8 (http://www.brenda-

enzymes.org/php/result_flat.php4?ecno=3.5.2.6&Suchword=&organism[]=Enterobacter+cloacae&show_tm=0).

In this study, all chemicals were prepared in 25 mM phosphate buffer (pH 7) to prevent pH alterations in the chemical solutions and avoid any potential negative effect on enzyme activity due to pH alteration.

During CD measurements, it is important to maintain the total absorbance of the sample (i.e., protein and buffer or solvent) within rational ranges to avoid excessive noise; therefore, it is crucial that samples must be free of optically active substances (e.g. optically active buffers and chemicals) (Miles and Wallace, 2006). The absorbance of the sample can be simply checked by measuring the High Tension (HT) voltage signal during a CD spectrum measurement of a sample (Kelly *et al*., 2005). High Tension voltage is the voltage applied to photomultiplier in order to amplify the signal. For many CD machines a nominal maximum value for HT is determined, values over this figure leading to distorted data. For the JASCO spectrometer used in this study it is recommended that the HT should be generally less than 600 V (Kelly et al., 2005). In the present work, the suitability of various concentrations of chemicals, being recognised as cell permeabilisation reagents and categorized as surfactants, chelator agents, chaotropic agents, and solvents, for CD studies was checked by running blank spectra to ensure that the absorbance of any citizen in the solution is not too high. It is evident from HT figures (Figs. 4.3 and 4.4) that chemicals such as 1% BAC, 1% CTAB and 2 M urea increase the beyond 600 V before the spectrum is complete. This has the effect of increasing the cut off wavelength (the lowest wavelength at which a spectrum can accurately be measured) preventing the recording of reliable CD data below 221 nm, 212 nm, and 206 nm, respectively. This, of course, is not a problem if changes in the CD signals at 222 nm are used to assess the unfolding of a protein.

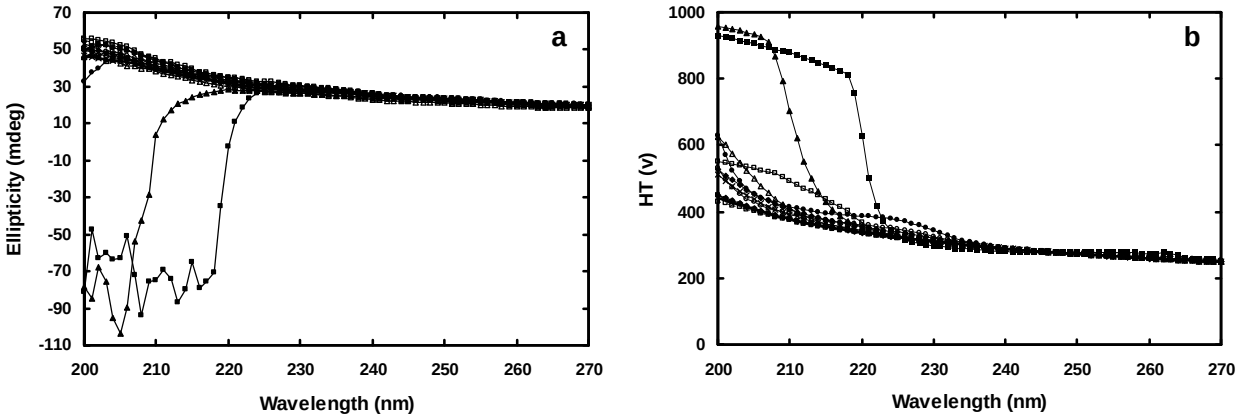

Fig. 4.3 CD spectra (a) and the corresponding HT signals (b) of various surfactants. Spectra were recorded in a 2 mm path length capillary cell on a Jasco J-810 spectropolarimeter at a scan speed of 100 nm/minute, response 2 s, and a bandwidth of 1 nm. Three scans were accumulated. Key: (—▫—) 0.1% BAC; (—▪—) 1% BAC; (—△—) 0.1% CTAB; (—▲—) 1% CTAB; (—+—) 1% SDS; (—×—) 1% DOC; (—○—) 0.05% Triton X-100; (—●—) 0.1% Triton X-100; (—◇—) 0.1% Tween 20; (—◆—) 1% Tween 20; (—▫—) 0.1% Brij; (—▪—) 1% Brij;(——) water.

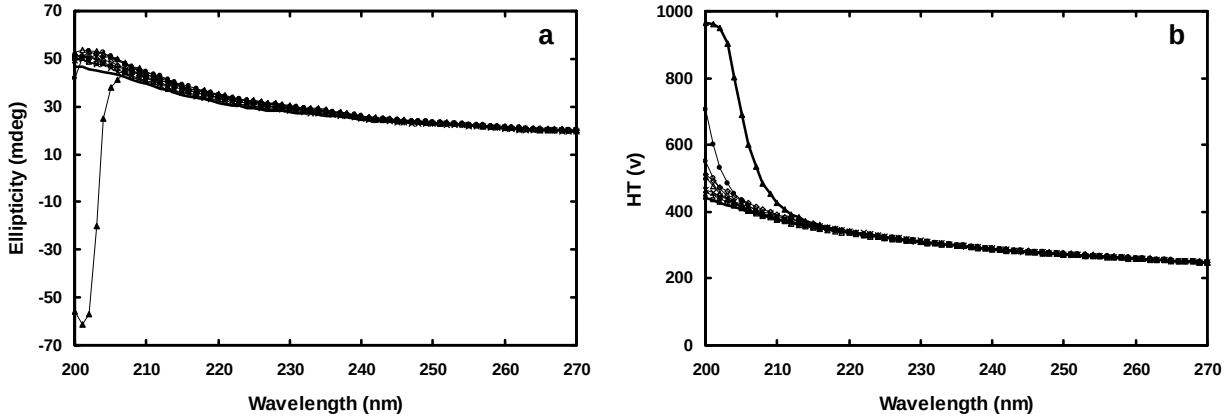

Fig. 4.4 CD spectra (a) and the corresponding HT signals (b) of various chelators and chaotropic agents. Spectra were recorded in a 2 mm path length capillary cell on a Jasco J-810 spectropolarimeter at a scan speed of 100 nm/min, response 2 s, and a bandwidth of 1 nm. Three scans were accumulated. Key: (—×—) 10 mM EDTA; (—+—) 10 mM EDA; (—▫—) 10 mM NTA; (—◇—) 1% SHMP; (—△—) 200 mM Urea; (—▲—) 2 M Urea; (—○—) 100 mM GndCl; (—●—) 200 mM GndCl; (——) water.

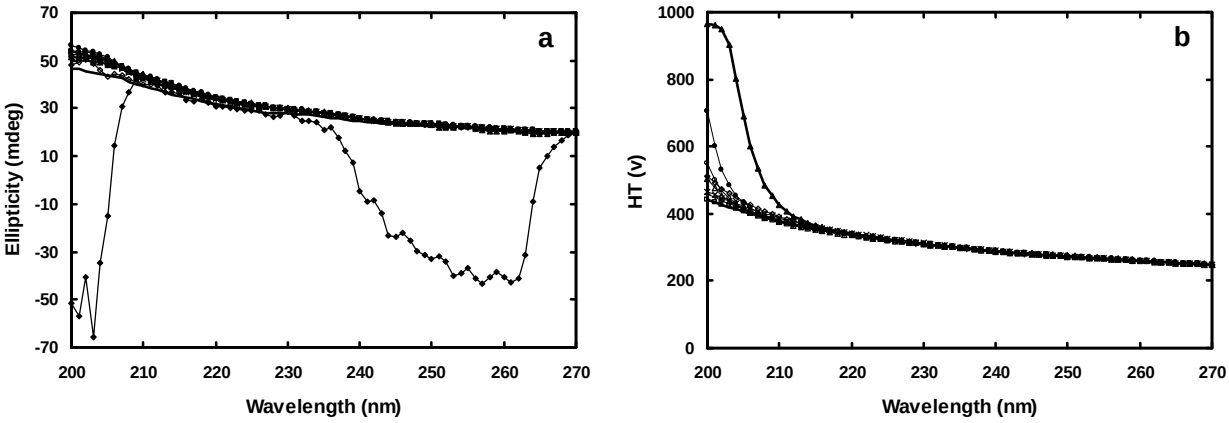

Fig. 4.5 CD spectra (a) and the corresponding HT signals (b) of various solvents. Spectra were recorded in a 2 mm path length capillary cell on a Jasco J-810 spectropolarimeter at a scan speed of 100 nm/min, response 2 s, and a bandwidth of 1 nm. Three scans were accumulated. Key: (—×—) 1% Hexane; (—+—) 1% Xylene; (—□—) 1% Toluene; (—◇—) 1% Benzene; (—△—) 1% Diethylether; (—○—) 1% Acetone; (—■—) 1% Chloroform; (—◆—) 1% Pyridine; (—▲—) 1% Ethanol; (—●—) 10% Isoamyl alcohol; (——) water.

One per cent pyridine had strong absorbance below 270 nm (Fig. 4.5), and could not be used in CD studies whatsoever. Apart from these, the other chemicals (at the concentrations showed in Figs. 4.3 to 4.5) did not cause excessive absorbance in UV-region, and deemed to be appropriate for CD studies.

After determining that the concentration of chemicals used in the study were complimentary for CD studies, the spectra of alpha-amylase (from *Asp. orizae*) and beta-lactamase (from *E. cloacae*), and Fab D1.3 (purified on HiTrap SP Sepharose XL/ HiTrap protein G HP column) at a concentration of 1 mg/mL in the presence of chemicals were measured. Figures 4.6 and 4.7 illustrate the changes in intrinsic CD spectra of beta-lactamase in the presence of different kinds of chemicals.

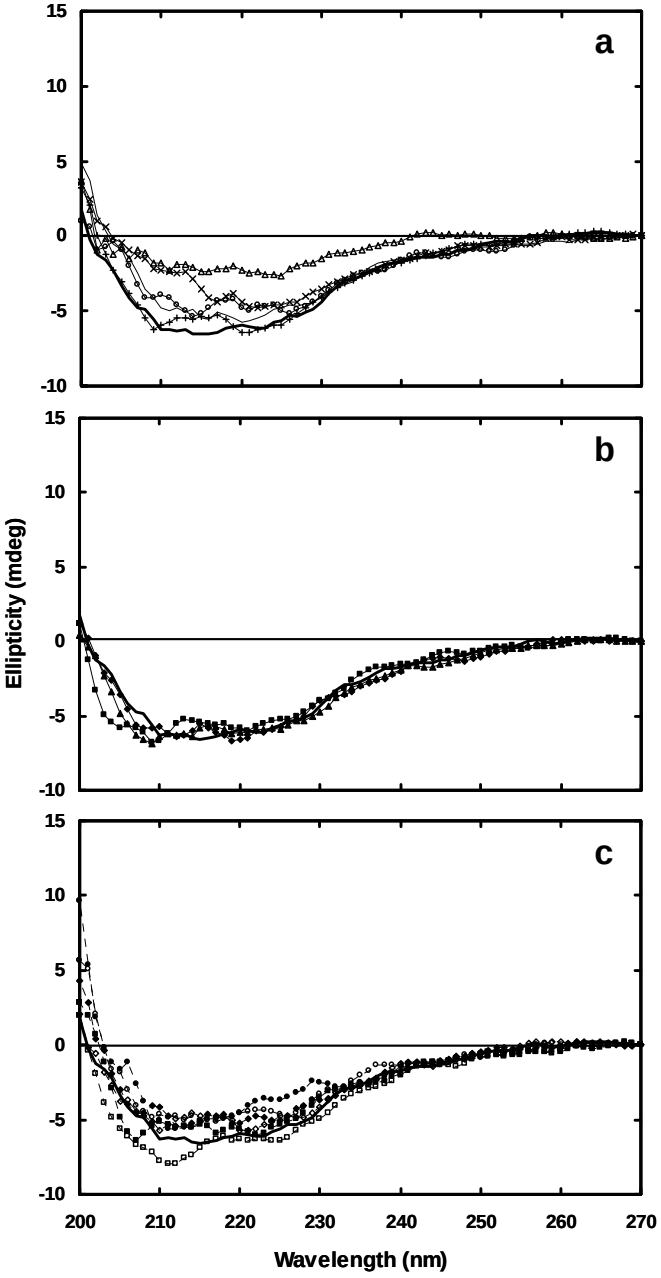

Fig. 4.6 Intrinsic CD spectra of beta-lactamase in the presence of (**a**): cationic surfactants; (**b**): anionic surfactants; and (**c**): non-ionic surfactants. Spectra were recorded at a protein concentration of 1 mg/mL in 25 mM sodium phosphate buffer (pH 7.5). Key: (—) 0.01% BAC; (—×—) 0.05% BAC; (—+—) 0.01% CTAB; (—○—) 0.05% CTAB; (—△—) 0.1% CTAB; (—■—) 0.1% SDS; (—◆—) 0.1% DOC; (—▲—) 1% DOC; (—○—) 0.05% Triton X-100; (—●—) 0.1% Triton X-100; (—◇—) 0.1% Tween 20; (—◆—) 1% Tween 20; (—□—) 0.1% Brij; (—■—) 1% Brij; (——) water.

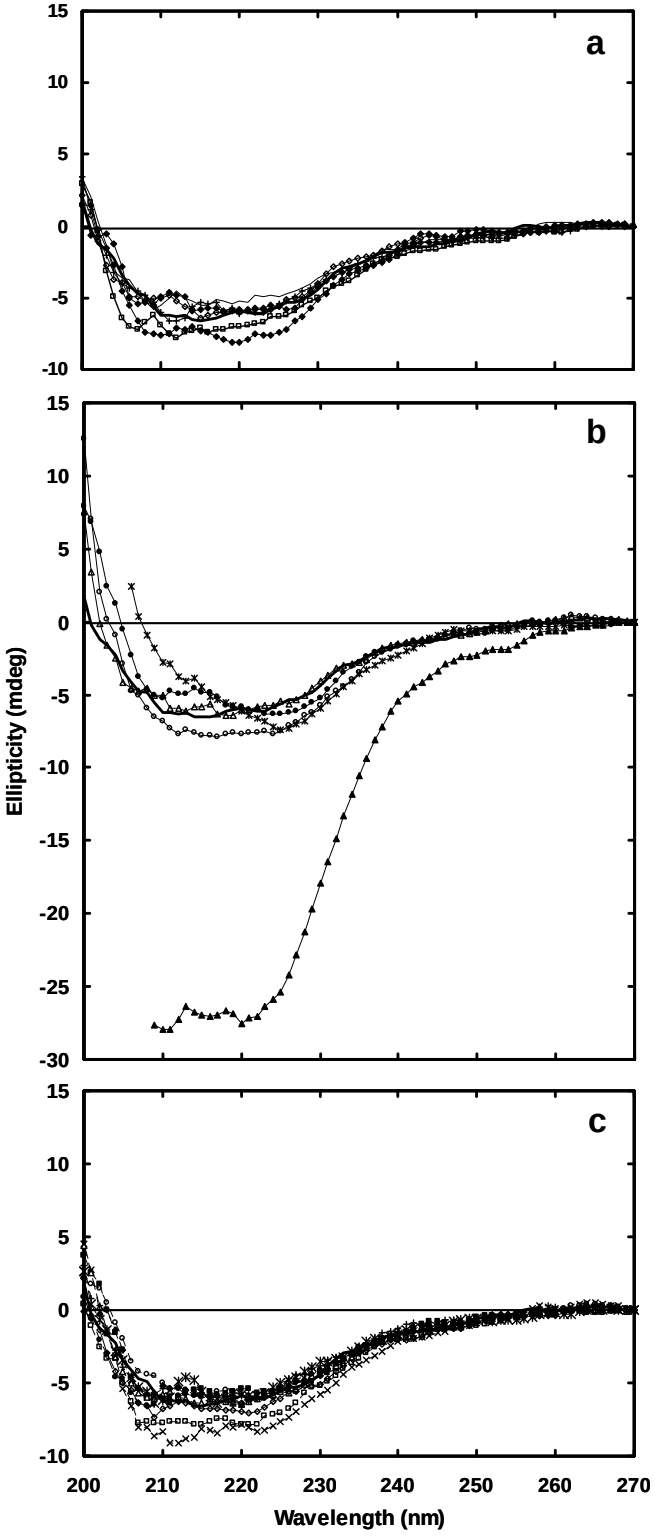

Fig. 4.7 Intrinsic CD spectra of beta-lactamase in the presence of (**a**): chelating agents; (**b**): chaotropic agents; and (**c**): solvents. Spectra were recorded at a protein concentration of 1 mg/mL in 25 mM sodium phosphate buffer (pH 7.5). CD spectra of the protein in the presence of 2 M urea and 500 mM GndCl were truncated below 208 nm and 206 nm, respectively, because of excessive noise (high HT) at these wavelengths. Key: (—) 10 mM EDTA; (—+—) 1 mM DEA ; (—×—) 10 mM DEA; (—▪—) 10 mM NTA; (—◇—) 0.1% SHMP; (—◆—) 1% SHMP; (—△—) 200 mM Urea; (—▲—) 2 M Urea; (—○—) 100 mM GndCl; (—●—) 200 mM GndCl; (—✱—) 500 mM GndCl; (—◆—) 1% Hexane; (—■—) 1% Xylene; (—△—) 1% Toluene; (—○—) 1% Benzene; (—✱—) 1% Diethylether; (—●—) 1% Acetone; (—+—) 1% Chloroform; (—×—) 1% Ethanol; (—◇—) 1% Isoamyl alcohol; (—▫—) 10% Isoamyl alcohol; (——) water.

The alterations in intrinsic CD spectra of alpha-amylase in the presence of chemicals have been shown in Figs. 4.8 and 4.9.

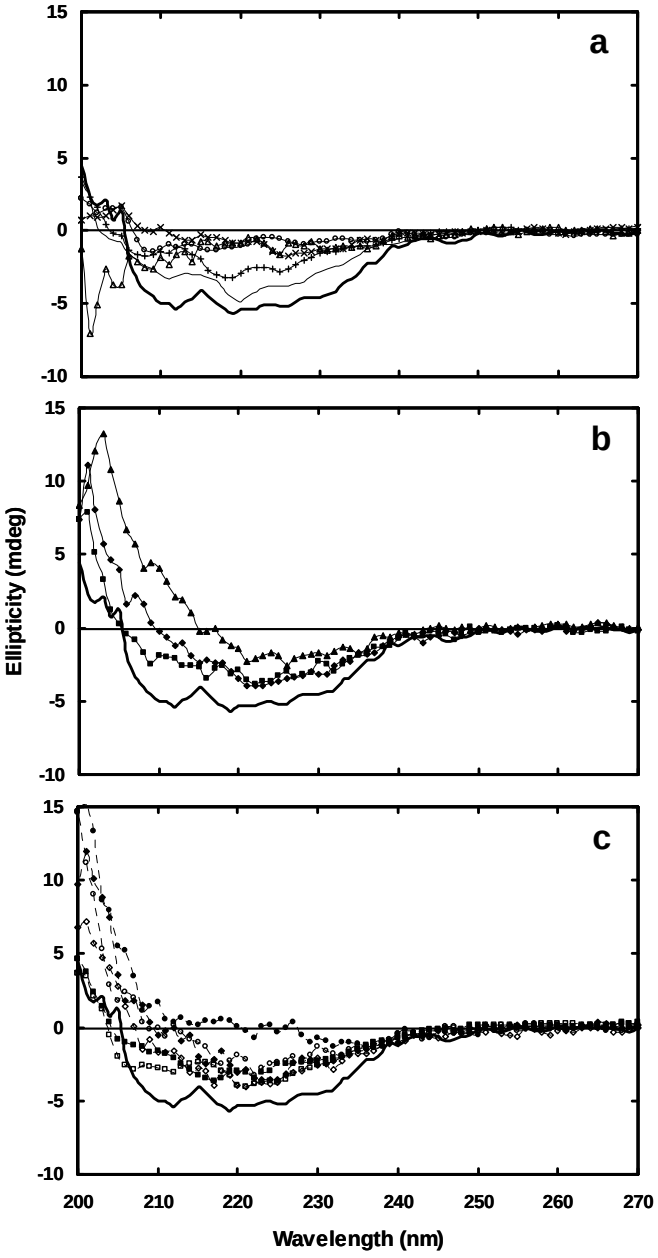

Fig. 4.8 Intrinsic CD spectra of alpha-amylase in the presence of (a): cationic surfactants; (b): anionic surfactants; and (c): non-ionic surfactants. Spectra were recorded at a protein concentration of 1 mg/mL in 25 mM sodium phosphate buffer (pH 7.5). Key: (—) 0.01% BAC; (—×—) 0.05% BAC; (—+—) 0.01% CTAB; (—○—) 0.05% CTAB; (—△—) 0.1% CTAB; (—■—) 0.1% SDS; (—◆—) 0.1% DOC; (—▲—) 1% DOC; (—○—) 0.05% Triton X-100; (—●—) 0.1% Triton X-100; (—◇—) 0.1% Tween 20; (—◆—) 1% Tween 20; (—□—) 0.1% Brij; (—■—) 1% Brij; (——) water.

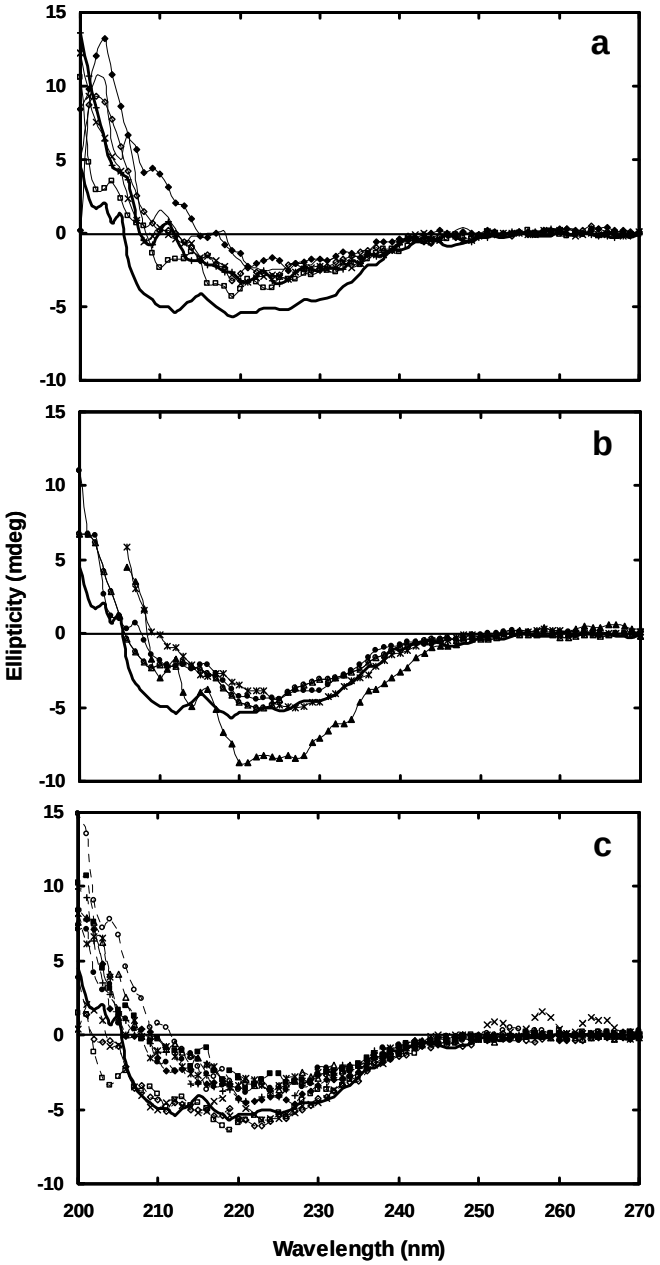

Fig. 4.9 Intrinsic CD spectra of alpha-amylase in the presence of (a): chelating agents; (b): chaotropic agents; and (c): solvents. Spectra were recorded at a protein concentration of 1 mg/mL in 25 mM sodium phosphate buffer (pH 7.5). CD spectra of the protein in the presence of 2 M urea and 500 mM GndCl were truncated below 206 nm because of excessive noise (high HT) at these wavelength. Key: (——) 10 mM EDTA; (—+—) 1 mM DEA ; (—×—) 10 mM DEA; (—▫—) 10 mM NTA; (—◇—) 0.1% SHMP; (—◆—) 1% SHMP; (—△—) 200 mM Urea; (—▲—) 2 M Urea; (—○—) 100 mM GndCl; (—●—) 200 mM GndCl; (—✱—) 500 mM GndCl; (—◆—) 1% Hexane; (—■—) 1% Xylene; (—△—) 1% Toluene; (—○—) 1% Benzene; (—✱—) 1% Diethylether; (—●—) 1% Acetone; (—+—) 1% Chloroform; (—×—) 1% Ethanol; (—◇—) 1% Isoamyl alcohol; (—▫—) 10% Isoamyl alcohol; (——) water.

Alpha-amylase and beta-lactamase are *α*-helix rich proteins, and the CD spectrum of *α*-helical protein takes over the region between 240 and 200 nm with two reasonably intense negative bands at about 222 and 208 nm (Miles and Wallace, 2006). To test the hypothesis that CD could act as a measure of protein fold a simple measure was devised that used the intensity of the CD spectrum at key wavelengths as an indicator of fold. In this case the intensity at 222 nm was used as this wavelength is thought to reflect the presence of alpha helix in the sample. Besides, activity of beta-lactamase and alpha-amylase in the presence of various chemicals was measured,

and correlation between the CD intensity at 222 nm and enzyme activity in the presence of each reagent was drawn (Figs. 4.10 and 4.11). Based on CD signals at 222 nm, certain chemicals such as 100 mM GndCl, 200 mM urea, 1% hexane, 1% chloroform and 1% ethanol caused trivial changes in the intrinsic CD spectra of the proteins, while chemicals such as 0.05% BAC and 0.1% CTAB led to large changes in the CD spectra of the proteins. Also it is evident that changes in the CD spectra increase with increasing the concentration of chemicals.

Electrostatic interactions between charged amino acids in a protein are important for the formation of its three dimensional structure and its function. Charged chemicals such as CTAB, BAC and DOC can attach to the charged amino acids in a protein and disrupt the electrostatic interactions within the protein, and as a result the active site will probably be lost. Organic solvent properties such as solvent hydrophobicity, hydrogen bonding capacity and miscibility in water have profound effect on the structural integrity of proteins. In hydrophobic water-immiscible solvents, water molecules tend to stay at the protein surface because of the solvophobic and hydrophilic nature of the protein surface. In contrast, polar solvents that can easily strip water from the surface of the protein and compete strongly for hydrogen bonds between protein atoms usually denature the structure to an unfolded state (Mattos and Ringe, 2001; Ru *et a*l., 1999). Alcohols, having some hydrophilic components, are moderate competitors for amide hydrogen bonds, and they tend to disrupt tertiary structure and leave secondary structure interactions undisrupted (Babu and Douglas, 2000; Knubovets *et al*., 1999, Mattos and Ringe, 2001).

As shown in Figs. 4.10 and 4.11, changes in the secondary structure of beta-lactamase and alpha amylase do not necessarily lead to loss of enzyme activity. For example, while 0.05% CTAB, 1% DOC, and 1% Brij caused substantial changes in both CD signals and in the activity of alpha-amylase, other chemicals such as 0.1% Triton, 1% SHMP, and 1% diethylether only led to a decrease in the intensity of CD signals but not in activity of the protein. Also there are examples where chemicals caused a marked reduction in enzyme activity but a small change in the intrinsic CD signal (see values for beta-lactamase in the presence of 1% DOC, 0.05% BAC, and 0.05% CTAB in Fig. 4.10). These discrepancies may indicate that the chemicals are altering the structure of the proteins in ways that are not recorded by CD. For

instance if the chemicals disrupt side chain or subunit packing without altering secondary structure then very little change would be detected in the CD spectrum. These results would therefore suggest that just such subtle changes are being induced by these chemicals. These discrepancies may also show that the altered structure detected by CD is outside the enzyme active site, in which case the enzyme activity would not be afflicted. Therefore, enzymes with insignificantly modified secondary structure may or may not have intrinsic activity depending on the site in which alteration is occurred. It is also seen in Figs. 4.10 and 4.11 that the changes in the CD signals and in the activities of beta-lactamase and alpha-amylase in the presence of same chemicals are not identical.

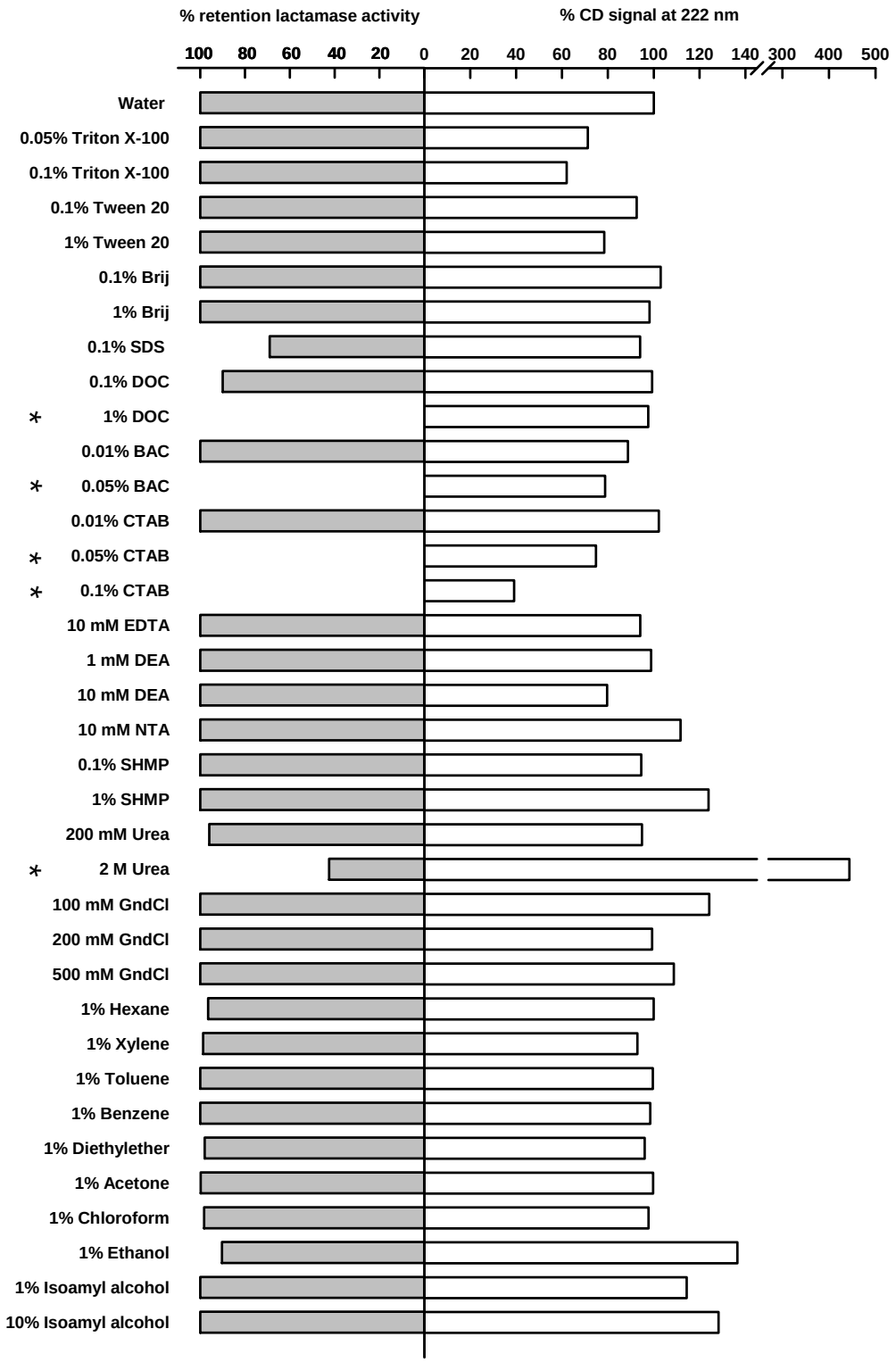

Fig. 4.10 Correlation of CD spectra changes and beta-lactamase activity in the presence of various chemicals. The growing discrepancies between CD and activity results have been shown by star.

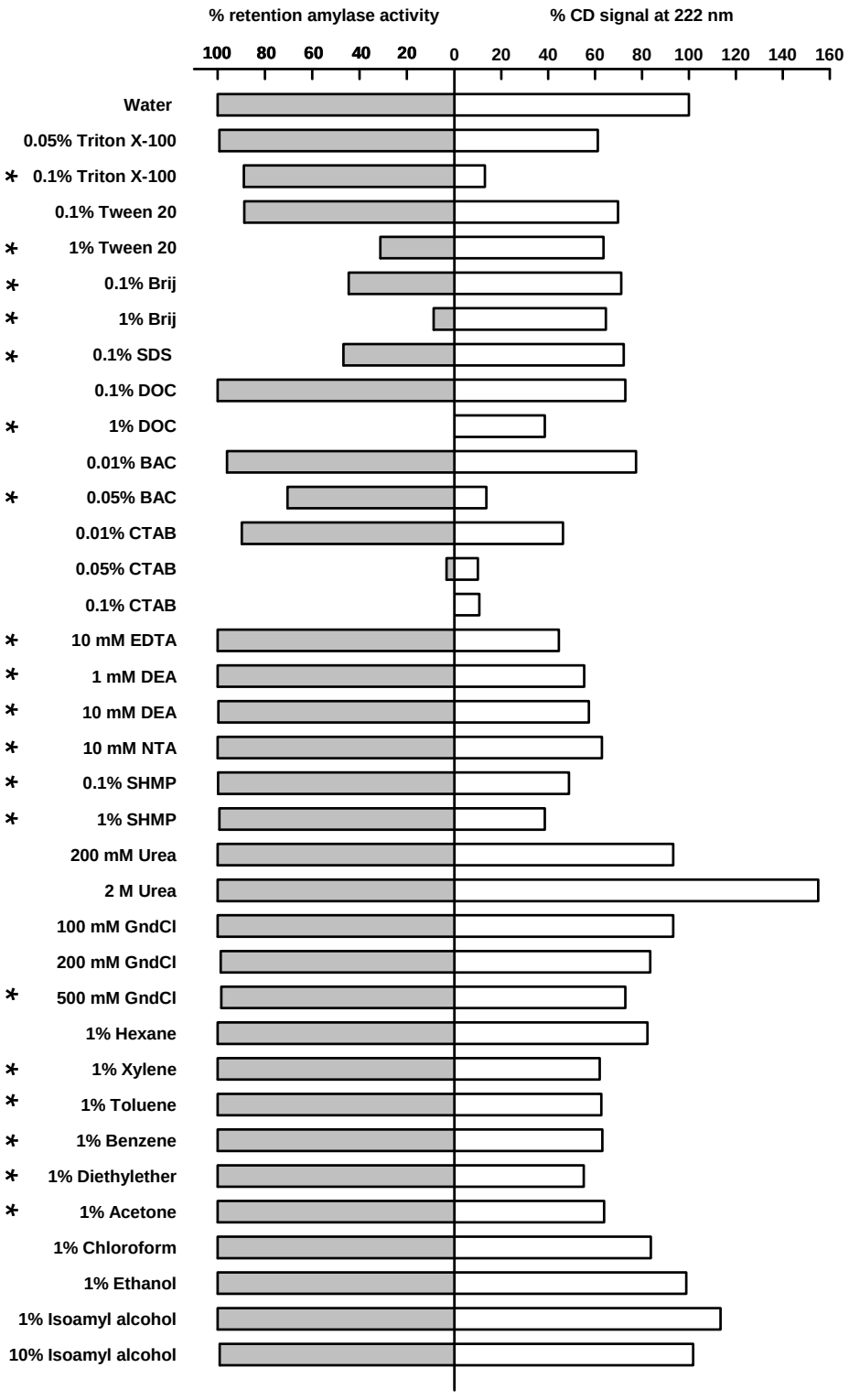

Fig. 4.11 Correlation of CD spectra changes and alpha-amylase activity in the presence of various chemicals. The growing discrepancies between CD and activity results have been shown by star.

Anomalously the CD signals of alpha-amylase and beta-lactamase, were greatly increased in the presence of 2 M urea, this indicates an overall increase in structure under these conditions. One potential explanation for this result would be that the protein is aggregating to form a species with an enhanced secondary structure. Such occurrences have been observed for proteins that form amyloid like fibres when partially denatured. To examine the potential that protein aggregation had occurred the size of the protein in solution using analytical ultracentrifugation (AUC) (Berkowitz, 2006; Liu *et al*., 2006) was examined. AUC studies showed that there was no aggregation of the enzymes in the presence of 2 M urea (Fig. 4.12); however, the location and intensity of alpha-amylase and beta-lactamase AUC peaks were changed when compared with the controls. Therefore, from results of sedimentation velocity experiments, it is construed that the increase in the proteins CD signals is not due to aggregation. It is also noticeable that no remarkable decrease in the enzyme activities observed in the presence of 2 M urea (Figs. 4.10 and 4.11).

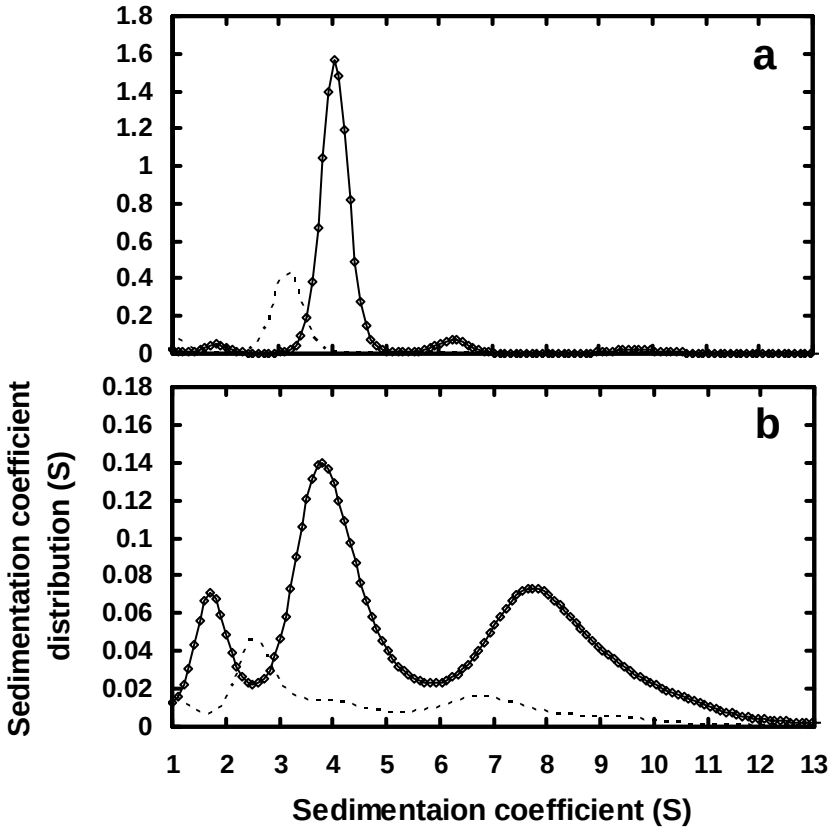

Fig. 4.12 Sedimentation experiment of alpha-amylase (a) and beta-lactamase (b) in the presence of urea. Key: (—◇—) 25 mM sodium phosphate buffer (pH 7.5); (- - - -) 2 M Urea/buffer

The effects of various chemical permeabilisation agents on the secondary structure of Fab D1.3 and on the Fab ability to bind to immobilized HEWL was also investigated. The CD spectra of Fab D1.3 at a concentration of 1 mg/mL and in the presence of various chemicals were measured in thhe same manner. Alteration in intrinsic CD spectra of Fab D1.3 in the presence of chemicals has been shown in Figs. 4.13 and 4.14. Fab D1.3 is *β*-sheet rich protein, the CD spectrum of which is characterised by a small negative band near 217 nm (Miles and Wallace, 2006); hence, the wavelength 214 nm was used to indicate the percentage of changes taken place in the CD signal as a result of alterations in the secondary structure of the protein. As a complementary test, the ability of Fab D1.3 to attach to HEWL in the presence of chemicals was also evaluated (Fig. 4.15). *β*-sheets give rise to considerably less intense signals than helices, and show more variations in spectral characteristics (Miles and Wallace, 2006). CD results from Fab D1.3 study (Figs. 4.13 and 4.14) are in agreement with this, and demonstrate that in the presence of chemicals (such as 0.1-1% Tween 20, 0.1-1% Brij, 0.01-0.05% BAC, 0.01-0.05% CTAB, 10 mM chelators, 200-500 mM GndCl, and 2 M urea) changes in CD signals of Fab D1.3 are greater than those of alpha-amylase and beta-lactamase (*α*-helix rich proteins), results of which have been previously shown (i.e. Figs. 4.6 to 4.9). However, Fab D1.3 retained its capability to bind to HEWL when in contact with chemicals – particularly 1% DOC, 0.05% CTAB and 0.05% BAC, in presence of which there was negligible or no alpha-amylase and beta-lactamase activity. This demonstrates that although Fab D1.3 shows more variations in spectral characteristics in the presence of chemicals, its ability to bind to HEWL remained greatly intact at such conditions.

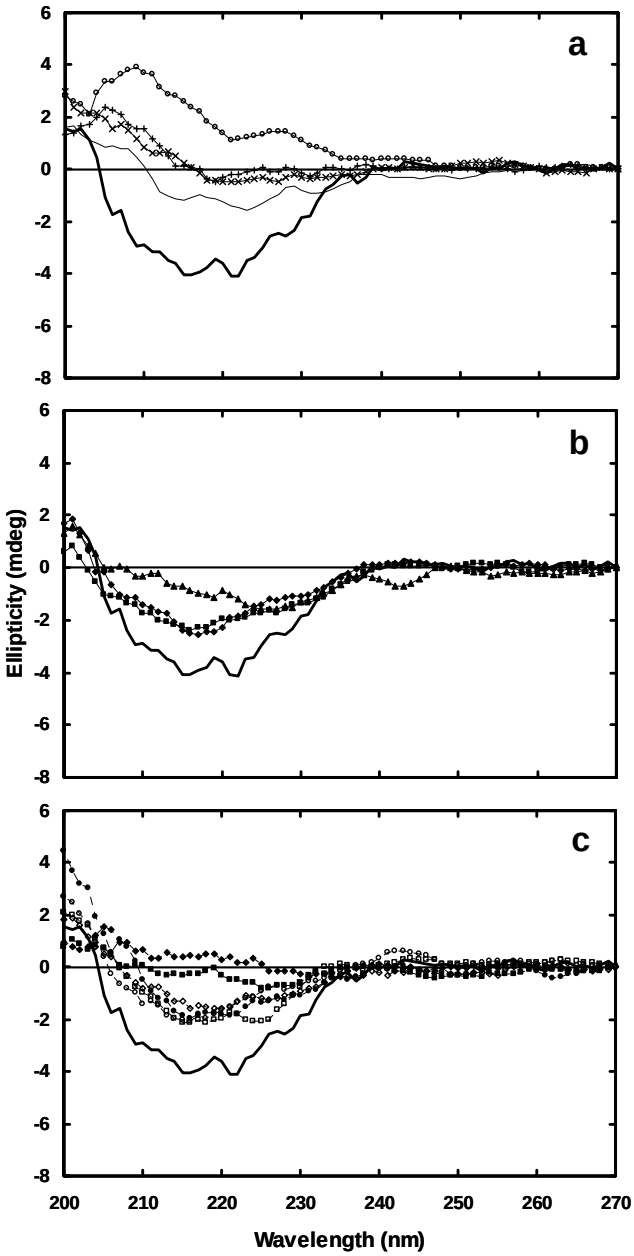

Fig. 4.13 Intrinsic CD spectra of Fab D1.3 in the presence of (**a**): cationic surfactants; (**b**): anionic surfactants; and (**c**): non-ionic surfactants. Spectra were recorded at a protein concentration of 1 mg/mL in 25 mM sodium phosphate buffer (pH 7.5). Key: (—) 0.01% BAC; (—×—) 0.05% BAC; (—+—) 0.01% CTAB; (—○—) 0.05% CTAB; (—■—) 0.1% SDS; (—◆—) 0.1% DOC; (—▲—) 1% DOC; (—○—) 0.05% Triton X-100; (—●—) 0.1% Triton X-100; (—◇—) 0.1% Tween 20; (—◆—) 1% Tween 20; (—□—) 0.1% Brij; (—■—) 1% Brij; (——) water.

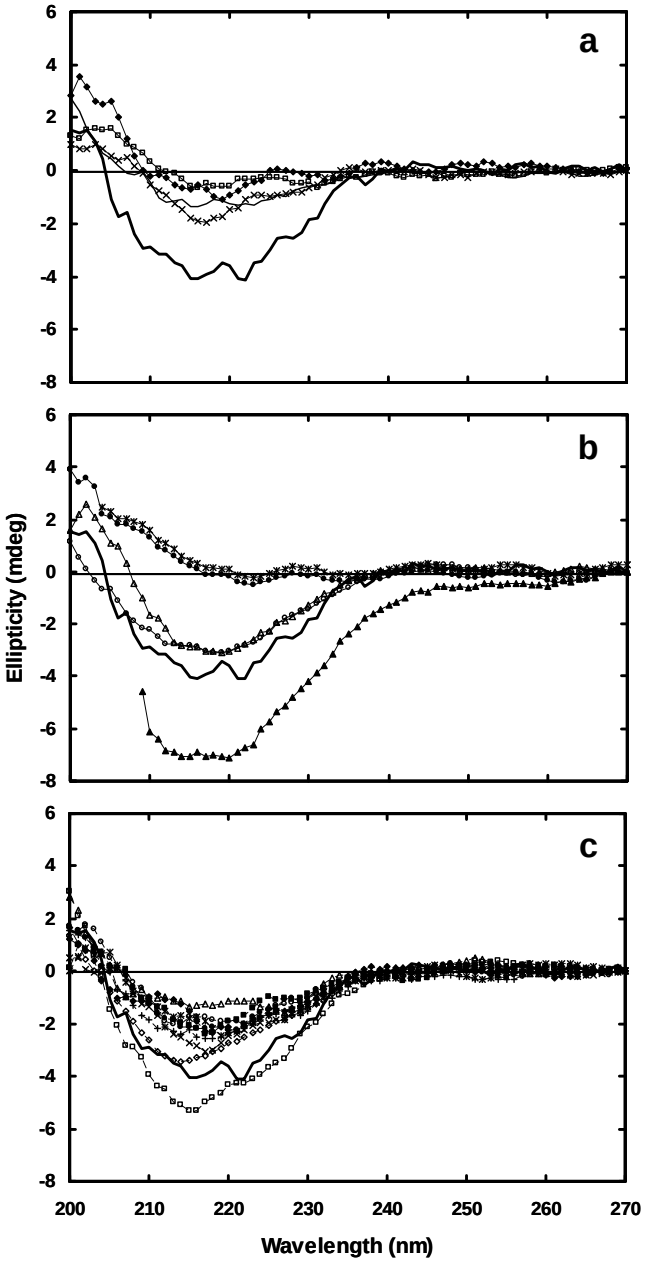

Fig. 4.14 Intrinsic CD spectra of Fab D1.3 in the presence of (**a**): chelating agents; (**b**): chaotropic agents; and (**c**): solvents. Spectra were recorded at a protein concentration of 1 mg/mL in 25 mM sodium phosphate buffer (pH 7.5). CD spectra of the protein in the presence of 2 M urea and 500 mM GndCl were truncated below 204 nm and 209 nm, respectively, because of excessive noise (high HT) at these wavelengths. Key: (——) 10 mM EDTA; (—×—) 10 mM DEA; (—▫—) 10 mM NTA; (—◆—) 1% SHMP; (—△—) 200 mM Urea; (—▲—) 2 M Urea; (—○—) 100 mM GndCl; (—●—) 200 mM GndCl; (—✱—) 500 mM GndCl; (—◆—) 1% Hexane; (—■—) 1% Xylene; (—△—) 1% Toluene; (—○—) 1% Benzene; (—✱—) 1% Diethylether; (—●—) 1% Acetone; (—+—) 1% Chloroform; (—×—) 1% Ethanol; (—◇—) 1% Isoamyl alcohol; (—▫—) 10% Isoamyl alcohol; (——) water.

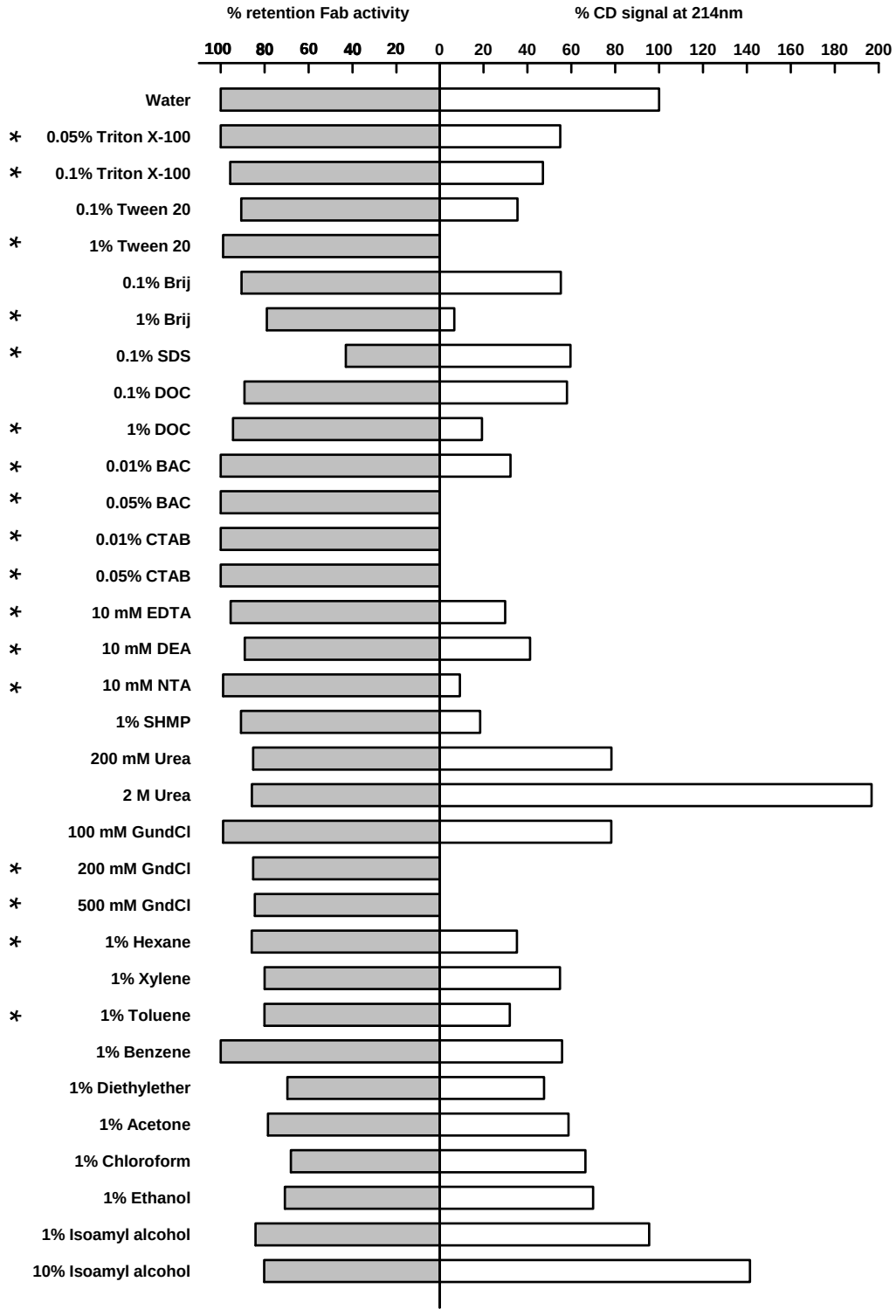

Fig. 4.15 Correlation of CD spectra changes and Fab D1.3 activity in the presence of various chemicals. The growing discrepancies between CD and activity results have been shown by star.

The CD signals of Fab D1.3 were increased in the presence of 2 M urea and 10% isoamyl alcohol, and shifted towards positive values of Ellipticity in the presence of chemicals such as 1% Tween 20, 1% Brij, and 0.05% CTAB. The formation of the Fab aggregates, which can be a potential explanation for such anomalous results, was investigated by AUC. AUC results have been shown in Fig. 4.16; it is evident that the intensity and location of the AUC peaks have been changed to different extents when compared with the control, and there was distinctive Fab aggregation (multimeration), being shown by increased number of sedimentation velocity peaks in the figure, in the presence of 0.05% CTAB; thus, the alteration in the shape and magnitude of the CD spectra in the presence of 0.05% CTAB (Fig 4.13) could be attributed to the protein aggregates. Other chemicals did not cause Fab D1.3 aggregation. It was impossible to study the Fab in the presence of 10% isoamyl alcohol by AUC, as 10% isoamyl alcohol solution was interfered with AUC.

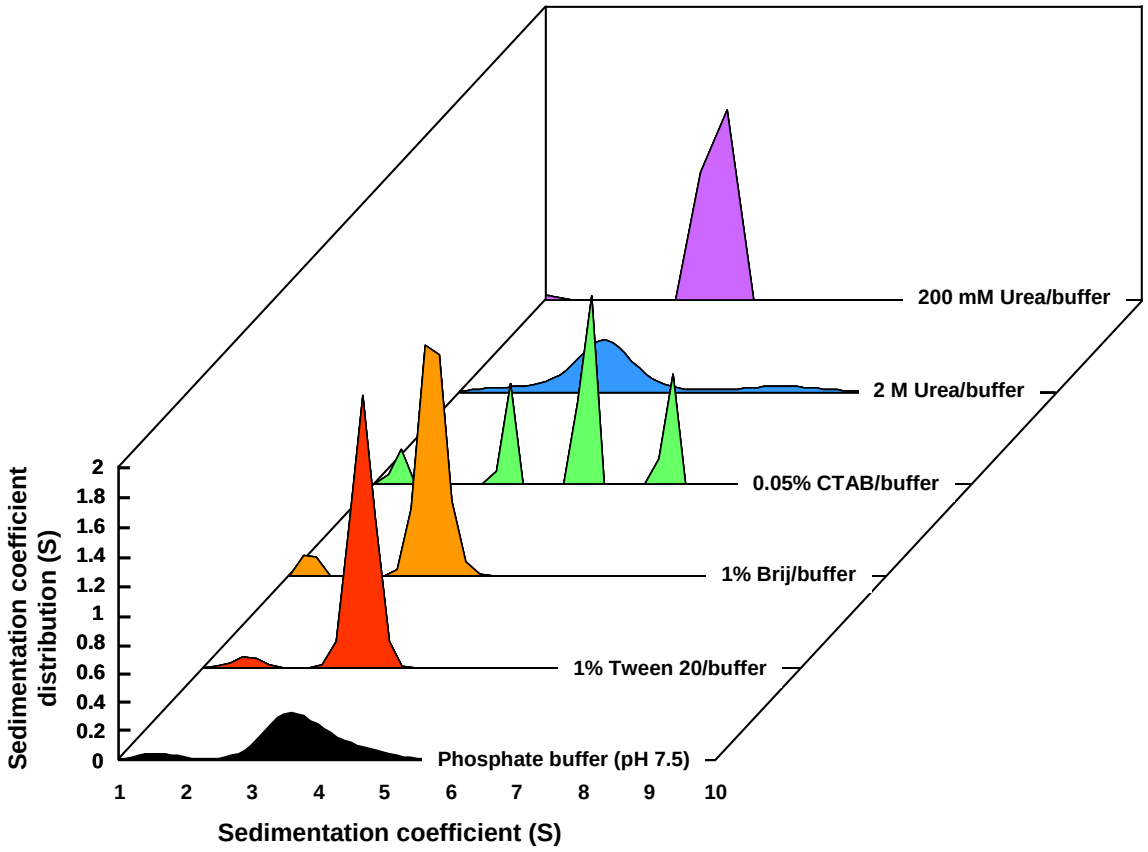

Fig. 4.16 Sedimentation velocity experiment of Fab D1.3 in the presence of chemicals.

4.4 Conclusions

In this chapter we have explored the question of what effect chemicals additives used in the purification process might have on the secondary structure and biological activity of three proteins: beta-lactamase, alpha-amylase and Fab D1.3. The effect of chemicals on the secondary structure of the target proteins was investigated through a high throughput circular dichroism (ht-CD), given the fact that the instrument is in effect at a prototype stage. In a general CD spectroscopy method, samples are analyzed by using quartz covetts which can have a range of path lengths. This conventional method can be very lengthy and cumbersome when a large number of samples are to be analyzed as the cuvett should be manually washed and cleaned after each measurement. This problem can be solved in a high throughput format in which sampling is done by an autosampler followed by injection into a capillary cell (2 mm path length) by an HPLC pump. The washing of the capillary cell in such system is automatically done by pumping distilled water or a solvent into the capillary cell prior to injection of the next sample. A large number of protein samples can be structurally analysed by such an ht-CD system within a short period of time. In the present study, the reproducibility of a newly developed high-throughput CD system was primarily confirmed by using BSA. Blank spectra of cell permeabilization reagents at various concentrations were recorded to recognize their suitability for CD studies. The blank CD spectra reported in this chapter will be also invaluable for those intending to study secondary structure alterations of proteins in the presence of these chemical reagents.

In the present work, the ht-CD system has been utilised as a tool in order to assess changes in the structure of three pure target proteins (i.e. beta-lactamase, alpha-amylase, and Fab D1.3) in the presence of various recognised cell permeabilisation reagents. The changes in the intrinsic CD spectra of the proteins in the far-UV region (approx. 240-200 nm) are evidence of perturbations in the secondary structure of proteins. Chemicals such as 0.05% BAC and 0.1% CTAB caused large changes in the intrinsic CD spectra of beta-lactamase and alpha-amylase. In Isoamyl alcohol (1 and 10%) and ethanol (1%), the spectra of beta lactamase and alpha amylase had a negative band greater than that of in water in the range from 200 to 240 nm. These

results are in agreement with Knubovet and colleagues (1999) studies in which they investigated the secondary structure of henn egg white lysozyme in solvents and observed a highly developed secondary structure and an increase in α-helix content of the protein in the presence of alcohol (i.e. methanol). Metalloproteins contain metal ions (e.g. beta-lactamase with Zn^{2+} ions and alpha-amylase with Ca^{2+} ions in their structure) which are important for the enzyme's activity and stability (Carenbauer *et al*., 2002; Gacar *et al*., 2005; Picao et al., 2008; Xu *et al*., 2008), and chelating agents sequester these ions from the proteins. Chelating agents in this study changed the secondary structure of alpha-amylase though the enzyme activity retained intact. However, they did not cause significant alteration in the secondary structure of beta-lactamase. According to the spectra recorded in the present work, the secondary structure of Fab D1.3 is more vulnerable than that of beta-lactamase and alpha-amylase to chemicals. The reason for this is that antibodies and their fragments are *β*-sheet rich proteins (Cathou *et al*., 1968; Doi and Jirgensons, 1970; Feige *et al*., 2010; Rehan and Younus, 2006) and changes in their CD signals are greater (Miles and Wallace, 2006) than *α*-helix rich proteins such as beta-lactamase and alpha amylase (Carenbauer *et a*l, 2002; Feller *et al*., 1999; Fitter and Haber-Pohlmeier, 2004).

After measuring the structural alterations by Ht-CD, the retention biological activity of the proteins in the presence of various chemicals has been determined. This was done to perceive whether the changes in the secondary structure of proteins lead to reduction in the biological activity. This investigation demonstrated that changes in the secondary structure of proteins do not necessarily lead to reductions in the biological activity, since such structural alterations may occur outside the region responsible for biological activity, or take place inside the active site(s) of proteins in a manner that the biological activity is retained. Concentration ranges of chemicals which do not influence biological activity of the target proteins in this study are considered safe and are employed in subsequent periplasmic release experiments using recombinant *E. coli* strains producing the same target proteins (Chapter 5).

4.5 References

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5 Chemical extraction of target proteins from the periplasm of recombinant *E. coli*

Abstract

The periplasm of *E. coli* has been known as a very suitable location for production of myriad recombinant proteins, particularly those having disulphide bonds in their structure; yet no generic periplasmic recovery methods with the potential for scale up exist. This work explains the development of chemical methods for the release of recombinant proteins from the periplasm of *E. coli*. Various chemicals, including detergents, chelators, chaotropes, and solvents were used singly and in different combinations to extract beta-lactamase, alpha-amylase, and Fab D1.3 expressed in the periplasm of *E. coli*. Prior to commencing main chemical permeabilisation experiments, washing bacterial cells with 50 mM Tris buffer pH 7.5 caused beta-lactamase and Fab D1.3 release (43% and 48%, respectively) from the periplasm. Amongst single chemicals made in 200 mM Tris buffer pH 7.5, sodium deoxycholate (0.025% to 0.1%) was very efficient generic permeabiliser, being able to release the target proteins from the periplasm more efficient than osmotic shock. It caused less total cell protein release and consequently higher enzyme specific activity, purification factor, and yield factor when compared to the osmotic shock disruption. EDTA (1 mM and 10 mM), 10 mM NTA and 0.1% SHMP extracted periplasmic Fab D1.3 as efficient as osmotic shock; however, they had low performance for releasing beta-lactamase and alpha-amylase. Ten per cent (v/v) isoamyl alcohol was exceptionally effective for periplasmic release of alpha-amylase (75%) and Fab D1.3 (168%), and caused very low amount of total protein release when compared to the osmotic shock treatment; nevertheless, the efficacy of 10% isoamyl alcohol for extracting beta-lactamase was low.

The release effectiveness of different combinations of chemicals was also investigated. It was shown that EDTA can increase the performance of chemicals such as 0.1% Brij, 0.1% SDS, 0.01% DOC, 0.1% Triton X-100, 0.01% CTAB, 0.01%

BAC and 200 mM urea for discharging beta-lactamase and/or Fab D1.3 from the periplasm. It was also demonstrated that 200 mM urea enhanced the efficiency of 0.1% Tween, 0.1% Brij, 0.1% SDS, 0.01% DOC, 0.01% CTAB and 0.01% BAC to release beta-lactamase and alpha-amylase. Combinations of solvents (hexane, xylene, benzene, toluene, and isoamyl alcohol) at 1% concentration with 0.025% DOC, 0.01 % CTAB, and 0.1% Triton X-100 could release the periplasmic alpha-amylase or Fab D1.3 up to 100%, and resulted in higher enzyme specific activity, purification factor and yield factor due to less total protein release, compared to osmotic shock.

5.1 Introduction

There are three locations for the manufacture of recombinant proteins in *E. coli*: the cytoplasm, the culture medium, and the periplasm. The cytoplasm of *E. coli* lacks the oxidative conditions required for high-level protein folding, particularly for proteins having disulphide bond(s) in their structures, and as a result production of high-level recombinant proteins in the cytoplasm of *E. coli* in many cases leads to inclusion bodies formation, which are insoluble aggregates of misfolded product (Carrió and Villaverde, 2002; Heebøll-Nielsen *et al.*, 2003; Ventura and Villaverde, 2006). Production of recombinant proteins through inclusion body formation pathway in the cytoplasm of *E. coli* is burdensome, as it needs extensive and expensive downstream operation units for the recovery of target protein in a fully refolded and biologically-active structure (Rudolph and Lilie, 1996; Singh and Panda, 2005; Vallejo and Rinas, 2004).

Directing protein to the extracellular medium (Mergulhao *et al*., 2005; Pugsley *et al*., 1997; Sandkvist, 2001) of *E. coli* is deemed advantageous; for example, minimum product contamination by host proteins occurs and also there is no need for cell disruption (Mergulhao *et al*., 2005). However, production of recombinant proteins in *E. coli* by secretion into culture medium has its own drawbacks. Considerable physical damages by shear forces of fermentation processes might happen, and highly diluted product requiring high volume capture is resulted in (Humphreys, 2007). Also, existence of the outer membrane in the structure of the cell envelope of

E. coli can be a barrier for secretion of recombinant proteins into the extracellular medium, and as a result proteins end up in the periplasm rather than the culture medium.

The periplasm of *E. coli*, on the other hand, is considered a very suitable site for the manufacture of recombinant proteins (Joly *et al.*, 1998; Laird *et al.*, 2005; Carter *et al.*, 1992; Chen *et al.*, 2004). Periplasmic disulphide bond forming (Dsb) proteins (Berkmen *et al*., 2007) , and periplasmic chaperones and isomerases (Betton, 2007; Richarme and Caldas, 1997) support the disulphide bond formation and accurate protein folding; hence, the periplasm is deemed a much better location for production of recombinant proteins having disulphide bonds in their structures. Additionally, the outer membrane is a permeable barrier, and the periplasm can be manipulated by being exposed to extracellular chemicals (≤ 600 Da) assisting protein folding (Barth *et al.*, 2000; Sandee *et al.*, 2005; Wunderlich and Glockshuber, 1993). The periplasm contains only 4-8% of the total cell protein (Park and Lee, 1998), and cytoplasmic nucleic acids and proteases – being challenging during product purification and recovery – are not present in the periplasm.

The potential of periplasmic expression systems has not been fully exploited at large scale, and as to our knowledge, no general method for selective release of periplasmically expressed proteins has been reported. The classical procedure for releasing proteins from the periplasmic space of *E. coli*, which is based on destabilisation of the outer envelope with EDTA and Lysozyme followed by cold osmotic shock (Neu and Heppel, 1965; Moir and Mao, 1990) without destroying the cytoplasmic membrane, is not feasible at large scale (Joly and Laird, 2007). The large volumes of liquid have to be handled at low temperatures if the shock is to be effective, and Lysozyme is expensive. Besides, this technique is time-consuming as an extended time is required to resuspend large amount of cells pellet. Thus, developing selective methods for release of periplasmically-located recombinant proteins is integral in order to exploit the advantages of the periplasmic expression systems for downstream processing.

The objective of this work was to study the permeabilisation of *E. coli* cells, producing recombinant proteins in the periplasm, using a wide range of chemicals. In order to develop reliable chemical methods for effective selective release of periplasmically expressed proteins, the effects of chemicals on the structure and activity of such proteins ought to be appraised, prior to applying them for protein release. In chapter 4, the impacts of a large number of acknowledged cell permeabilisation chemical reagents on the secondary structure and activity of beta-lactamase, alpha-amylase, and Fab D1.3 were investigated, and 'safe' chemical concentrations for cell permeabilisation were identified. In this chapter, extraction of target proteins (i.e. beta-lactamase, alpha-amylase, and Fab D1.3) by single and combination of 'safe' reagents from the periplasm of recombinant *E. coli* was studied.

5.2 Materials and Methods

5.2.1 Materials

E. coli CLD048, expressing the antibody fragment Fab D1.3 in the periplasm was obtained from Merck Sharp and Dohme Ltd. (Billingham, UK). *E. coli* JM107 PQR126 producing alpha-amylase in the periplasm, and *E. coli* JM109 producing beta-lactamase were received as gifts from Professor John Ward (Research Department of Structural & Molecular Biology, University College London, UK), and Dr. Tim Overton (School of Chemical Engineering, University of Birmingham, UK) respectively. Nutrient agar and nutrient broth were purchased from Oxoid (Basingstoke, UK). Yeast extract and tryptone were obtained from Becton, Dickinson and Co. (BD) (Sparks, MD, USA). Potato starch, kanamycin, ampicillin, tetracycline, sodium chloride, sodium deoxycholate (DOC), cetyltrimethylammonium bromide (CTAB), benzalkonium chloride (BAC), Triton X-100, Tween 20, Brij-35, ethylenediaminetetraacetic acid (EDTA), diethanolamine (DEA), nitriolotriacetic acid (NTA), sodium hexametaphosphate (SHMP), urea, guanidinium chloride (GndCl), diethyl ether, pyridine, isoamyl alcohol, sucrose, (hydroxymethyl)aminomethane (Tris), sodium phosphate monobasic, sodium phosphate dibasic, sodium carbonate, sodium hydrogen carbonate, glycine for electrophoresis, dialysis tubing cellulose membrane, chicken egg white Lysozyme (HEWL), RNase A (pancreatic), bovine

serum albumin (BSA), sodium salt of benzylpenicillin, and anti-human IgG (Fab specific) - peroxidise antibody produced in goat were obtained from Sigma-Aldrich (Gillingham, UK). Sodium dodecyl sulphate (SDS) was supplied by Bio-Rad Laboratories (Hercules, CA, USA). Ammonium hydroxide, phosphoric acid, magnesium sulphate, calcium chloride dehydrate, manganese (II) sulphate fourhydrate, hexane, xylene, chloroform, ethanol, acetone, glacial acetic acid and potassium iodide were obtained from Fisher Scientific (Loughborough, UK). Benzene, toluene and iodine were supplied by BDH Laboratory supplies (Poole, UK). TMB microwell peroxidase substrate (2-C) was purchased from KPL (Gaithersburg, MD, USA). IPTG was purchased from Melford Laboratories (Suffolk, UK).

5.2.2 Methods

5.2.2.1 Agar plate cultures

E. coli JM107 PQR126 cells were grown on nutrient agar plates complemented with 1% (w/v) potato starch and 20 µg/mL kanamycin. Transformed *E. coli* K12 JM109 cells were propagated on nutrient agar containing 100 µg/mL ampicillin. *E. coli* CLD048 cells from the cell bank, stored at -80°C, were propagated overnight on Nutrient agar supplemented with 15 mg/L tetracycline.

5.2.2.2 Shake Flask cultivation for the production of beta-lactamase

For this purpose, a single colony of *E. coli* JM109 was added to 100 mL Luria-Bertani (LB) (comprising 1% w/v NaCl, 0.5% yeast extract, 1% tryptone) supplemented with 100 µg/mL ampicillin and incubated overnight (13 h) at 37°C in an orbital shaker at 200 rpm. Flasks (1 L) containing 200 mL LB and 100 µg/mL ampicillin were inoculated with 0.2 mL (0.1% v/v) of the 13 h preculture and placed on orbital shaker at 200 rpm and 37°C for 18 h. The cells were collected by centrifugation at 4,120 g for 600 s, and the pellets were frozen at -20°C for further cell fractionation experiments.

5.2.2.3 Shake Flask cultivation for the production of alpha-amylase

For this purpose, a single colony of *E. coli* JM107 PQR126 was suspended into 100 mL nutrient broth containing 10 µg/mL kanamycin and incubated overnight (13 h) at 200 rpm and 37°C. Flask (1000 mL) containing 200 mL nutrient broth supplemented with 10 µg/mL kanamycin was inoculated with 8 mL (4% v/v) of the 13 h preculture and placed on orbital shaker at 200 rpm and 37°C for 18 h. The cells were then harvested by centrifugation (Jouan C 422 centrifuge, SelectScience Ltd., Bath, UK) at 4,120 g for 600 s, and the pellets were frozen at -20°C for further cell fractionation experiments.

5.2.2.4 Shake-flask cultivation for the production of Fab D1.3

The complex medium employed for fed-batch fermentation (chapter 2, section 2.2.2.1) was used for shake-flask cultivation in order to obtain cells for permeabilisation experiments. Starting cultures were prepared by inoculating 100 mL aliquots of Luria Bertani (LB) broth (comprising 1% NaCl, 0.5% yeast extract and 1% tryptone) containing 15 mg/L tetracycline with a fresh single colony of *E. coli* CLD048 and shaking at 37°C and 200 rpm for 13 h. Eight millilitres of the 13 h LB broth preculture was added to 2 L Erlenmeyer flask containing 200 mL of the complex medium (i.e. as a 4% inoculum), and incubated at 37°C and 200 rpm. Fab D1.3 expression was induced by the addition of IPTG to a final concentration of 0.1 mM after 6.5 h cultivation (OD_{600nm} of ~10) and the culture left at 30°C for another 4.5 h, with shaking at 200 rpm. After 11 h (i.e. 4.5 h post induction), the bacterial cells were harvested by centrifugation (Jouan C 422 centrifuge, SelectScience Ltd., Bath, UK) at 4,120 g for 600 s, and the pellets were frozen at -20°C for further cell fractionation experiments.

For all three strains mentioned above, the resulting supernatants after centrifugation (i.e. culture broth) were analysed by respective analytical method to detect product release during shake flask culture.

5.2.3 Cell fractionation

Cell pastes were defrosted at room temperature and then resuspended in 50 mM Tris buffer pH 7.5, to a final OD_{600nm} of ~5. Aliquots (1 mL) were then centrifuged for 300 s at 15,800 g using an Eppendorf microfuge (5412 D, Eppendorf, Hamburg, Germany). The supernatants were removed and stored at -20°C to be analysed for product release, whilst the cell pellets were subjected to various chemical permeabilisation and mechanical disruption treatments as described below.

5.2.3.1 Osmotic shock treatment

5.2.3.1.1 Osmotic shock treatment for cells producing beta-lactamase and alpha-amylase

Classic osmotic shock method

Routinely, the cell pellets were re-suspended in 1 mL of 200 mM Tris buffer pH 7.5 containing 1 mM EDTA, 20% sucrose and 500 µg/mL Lysozyme. Following a brief incubation (0.25 h) at room temperature, the cells were harvested by centrifugation at 15,800 g for 300 s, and the supernatants were kept frozen at -20°C for further enzyme activity assay. The obtained cell pellet was resuspended in 1 mL cold distilled water and harvested at 15,800 g for 300 s, subsequent 0.25 h static incubation. All obtained supernatants were kept frozen at -20°C for further enzyme activity assay.

Modified osmotic shock method

French and colleagues (1996) have reported a different osmotic shock procedure in which cold distilled water is added to the osmotic shock solution (200 mM Tris buffer pH 7.5 containing 1 mM EDTA, 20% sucrose and 500 µg/mL Lysozyme) after 0.25 h incubation at room temperature. For conducting this modified osmotic shock treatment in the present work, the bacterial cell pellet of each strain in each

eppendorf tube was resuspended in 0.5 mL of 200 mM Tris buffer pH 7.5 containing 1 mM EDTA, 20% sucrose and 500 µg/mL Lysozyme. The tubes were then incubated statically 0.25 h at room temperature, and 0.5 mL cold distilled water was next added. After 0.25 h static incubation, the pellet was separated from supernatant by centrifugation at 15,800 g for 300 s. The supernatants containing the periplasmic proteins were kept frozen at -20°C for further enzyme activity assay.

5.2.3.1.2. Osmotic shock treatment for cells producing Fab D1.3

The osmotic shock treatment employed was recommended by Merck Sharp and Dohme Ltd. Routinely, the cell pellets were resuspended in 1 mL of 20 mM Tris buffer pH 8.0 supplemented with 2.5 mM EDTA and 20% sucrose (osmotic solution 1, OS_1). After a static incubation on ice for 600 s, the cells were harvested by centrifugation at 15,800 g for 120 s, and the supernatants were immediately frozen at -20°C for further Fab D1.3 analysis. The obtained cell pellet was resuspended in 1 mL of a second osmotic shock solution (designated OS_2) lacking sucrose (i.e. 20 mM Tris buffer pH 8.0 containing 2.5 mM EDTA), incubated on ice for 600 s, and centrifuged at 15,800 g for 120 s. The obtained supernatants were stored at -20°C until required.

5.2.3.2 High pressure homogenization

Bacterial cell pellets were resuspended in 50 mL of 200 mM Tris buffer pH 7.5, to a final OD_{600nm} of ~5, and then 5 mg RNase A was added to the mixture. The cells were subsequently disrupted using a continuous flow TS-Series benchtop disrupter (Constant Systems Ltd, Low March, UK) operated at 2.4 Bar and 5°C. The supernatant was separated from the cell debris by centrifugation at 15,800 g for 300 s, and stored at -20°C for further analyses.

5.2.3.3 Chemical permeabilisation

Three categories of chemicals (singly and in various combinations) were used for cell perturbation (see Table 5.1): surfactants; chelators & chaotropic agents; and organic

solvents. Prior to chemical treatment, the cell pellets were washed once with 50 mM Tris buffer pH 7.5, and then re-suspended in 1 mL of the chosen chemical. Following incubation for 1 h at room temperature with brief intermittent vortexing (Clifton Cyclone, Scientific Laboratory Supplies, Notingham, UK) every 600 s, the supernatants were separated from the cells by centrifugation at 15,800 g for 300 s and frozen at -20°C for further analyses.

Table 5.1 Chemicals utilised in cell permeabilisation experiment.

Surfactants			**Chelators and Chaotropic agents**	**Organic solvents**
Anionic	**Cationic**	**Non-ionic**		
DOC	CTAB	Triton X-100	EDTA	Hexane
SDS	BAC	Tween 20	DEA	Benzene
		Brij 35	NTA	Toluene
			SHMP	Diethyl ether
			Urea	Chloroform
			GndCl	Ethanol
				Acetone
				Pyridine
				Isoamyl alcohol

5.2.4 Analytical techniques

5.2.4.1 Enzyme assays and Fab D1.3 quantification

Beta-lactamase and alpha-amylase quantifications were done according to the technique described in Chapter 4 (sections 4.2.2.3 and 4.2.2.4). The amount of beta-lactamase in each sample was calculated using beta-lactamase from *Enterobacter cloacae* as a standard (concentration range: 0.1-1 Units per mL of 200 mM Tris buffer, pH 7.5). The amount of alpha-amylase in each sample was calculated using

alpha-amylase from *Aspergillus orizae* as a standard (concentration range: 1-10 Units per mL of 200 mM Tris pH 7.5 buffer)**.** ELISA described in Chapter 2 (section 2.2.2.4.2) was used for Fab D1.3 quantification.

5.2.4.2 Total protein quantification

The amount of total protein present in the samples was measured using bicinchoninic acid assay (BCA assay kit, Thermoscientific, Rockford, IL, USA) employing bovine serum albumin (BSA) as a standard.

5.3 Results and Discussion

Analysis of the broth culture media at the end of shake flask cultivation of the strains producing recombinant periplasmic proteins (i.e. beta-lactamase, alpha-amylase, and Fab D1.3) showed that none of the target proteins secreted into the extracellular medium. After shake flask cultivations, *E. coli* cells were harvested and washed with 50 mM Tris buffer pH 7.5 prior to chemical permeabilisation. Washing cells with 50 mM Tris buffer pH 7.5 could result in product release from the periplasm, and it was demonstrated that the amounts of product release by this washing step were not the same in different recombinant strains producing various periplasmic proteins. Washing cells with 50 mM Tris buffer pH 7.5 released 43% and 48% of the periplasmic beta-lactamase and Fab D1.3, respectively, whilst this cell washing step released only 1% of the periplasmic alpha-amylase (Figs 5.1 to 5.3). This can show that alpha amylase is more difficult to release from the periplasm than beta-lactamase and Fab D1.3. It has been previously reported that Tris at moderately high concentrations (e.g. up to 200 mM) can permeabilise the outer membrane, by interacting with LPS and replacing stabilizing cations, and sensitize gram negative bacteria to other agents such as Lysozyme, detergents and antibiotics. High concentration of Tris alone (100 mM, pH 7.2), without EDTA has been shown to release 20% of LPS from smooth *S. typhimurium.* (Hancock, 1984; Nikaido and Vaara, 1985; Vaara, 1992). In the present work, it was shown that Tris could individually release the periplasmic proteins in disparate extents.

Classic and modified osmotic shock procedures were used in order to recover beta-lactamase and alpha-amylase from the periplasm of bacterial cell. Beta-lactamase and alpha-amylase recovery figures obtained from the modified osmotic shock treatment were respectively 11% and 21% higher than those achieved from the classic osmotic shock (Figs 5.1 and 5.2). The efficiency of the modified osmotic shock in releasing periplasmic beta-lactamase and alpha-amylase was similar to the performance of homogenization. As mentioned earlier, in the modified osmotic shock treatment cold water is added to the osmotic shock solution (EDTA/sucrose/Lysozyme) containing bacterial cells after 0.25 h incubation at room temperature. This method has been previously employed to increase alpha-amylase recovery from the periplasm of *E. coli* PQR126 cells (French *et al*., 1996). It has been demonstrated that a rapid influx of water into the periplasm can expand the periplasmic space and increase the distance between the polysaccharide chains of peptidoglycan which are stacked together; subsequently, this can increase the binding of Lysozyme to the peptidoglycan matrix and assist cell disruption (French *et al*., 1996; Witholt *et al*., 1976).

For recovery of Fab D1.3 from the periplasm by osmotic shock, it was not feasible to include HEWL in the osmotic shock solution because HEWL is specific antigen for Fab D1.3, and including it in the osmotic shock solution compromises the Fab assay by ELISA, being based on attachment of Fab D1.3 to immobilised HEWL on microplate. Even in the absence of HEWL, osmotic shock was very efficient for releasing Fab D1.3 from the periplasm (Fig 5.3).

Various concentrations of chemicals were used to permeabilise *E. coli* cells and to extract beta-lactamase, alpha-amylase, and Fab D1.3. These chemicals were screened by ht-CD and activity assay experiments explained in Chapter 4. By sequence similarity searching against protein databases using the FASTA program (http://www.ebi.ac.uk/Tools/fasta33/), before conducting CD and activity assay experiments, it was shown that these enzymes were genetically most similar to the enzyme produced in the periplasm of *E. coli* JM107 PQR126 and *E. coli* K12 JM109 transformed with the cloning vector pBR322; there was 25.9% identity (51.2% similarity) between alpha amylase *Asp. orizae* and the alpha-amylase produced in *E.*

coli, and 33.1% identity (69.8% similarity) between beta lactamase *E. cloacae* and the beta- lactamase produced in *E. coli.*

Three categories of chemicals, all prepared in 200 mM Tris buffer pH 7.5, were singly utilized for bacterial cell permeabilisation in this study. Detergents (nonionic, anionic, and cationic), the first category of chemicals, resulted in different extent of product release from the periplasm of three bacterial strains in this study (Figs. 5.1 to 5.3). It is evident from the results that sodium deoxycholate at concentrations ranged from 0.025% to 0.1% is very efficient permeabiliser amongst the experimented detergents. However, increasing concentration of this anionic detergent resulted in a gradual rise in total protein release from the bacterial cells and subsequently lowered specific activity, purification factor and yield factor (Tables 5.2 and 5.3). However, total cell protein released by the highest concentration of DOC (i.e. 0.1%) was less than that released by osmotic shock, and as a result specific activity, purification factor and yield factor values obtained from cell permeabilisation by 1% (and lower concentrations) DOC were much higher than those acquired by osmotic shock. Increasing concentration of DOC from 0.05% to 0.1% did not enhance beta-lactamase discharge from the periplasm; also, there was no rise in D1.3 Fab release at concentrations above 0.025% of DOC. However, more alpha-amylase was discharged by higher concentration of DOC. These results show that Fab D1.3 and beta-lactamase are easier to release by DOC. Mechanism(s) by which DOC permeabilises the OM and releases the periplasmic proteins in a higher rate than other experimented detergents is not known.

It is apparent from the results that increasing the concentration of cationic detergents (CTAB and BAC) from 0.01% to 0.05% decreased the product release from the periplasm. This is due to negative impact of the cationic detergents on the structure and activity of the proteins. Circular dichroism and biological activity analyses (Chapter 4) of the proteins (i.e. beta-lactamase, alpha-amylae and Fab D1.3) demonstrated that there were substantial protein secondary structure alterations and loss of activity in the presence of increasing concentrations of CTAB and BAC. Also sedimentation velocity studies (Chapter 4) confirmed the formation of Fab D1.3 multimers (aggregates) in the presence of 0.05% CTAB. Bansal- Mutalik and Gaikar

(2003) have also reported that treatment of *E. coli* cells with aqueous CTAB solutions (10 mM) caused substantial penicillin acylase release, but the enzyme activity recovered was only 18% due to deactivation of the enzyme by CTAB. Therefore, cationic detergents do not look conducive for *E. coli* cell permeabilisation because of their detrimental impacts on protein structure and activity.

As illustrated in the figures 5.1 to 5.3, the most efficient non-ionic detergents could release maximum 25 to 40 per cent of the periplasmic proteins, when compared to osmotic shock treatment. It is known that the outer membrane of gram-negative bacteria is resistant to non-ionic and anionic detergents, but sensitive to cationic detergents which bind to the negatively charged LPS and phospholipids in the cell envelope (Bansal-Mutalik and Gaikar, 2003; Burman *et al*., 1972; D'mello and Yotis, 1987; Filip *et al*., 1973; Vaara, 1992). The main specificity of the detergents is for phospholipids and the proteins in the cytoplasmic membrane, modifying of which leads to the discharge of cytoplasmic proteins. However, neutral and anionic detergents may cause disruption of gram-negative bacterial cells provided that the cells are pretreated (e.g., by EDTA) (Middelberg, 1995). It can be construed that 200 mM Tris buffer pH 7.5 utilized in this work destabilized the OM of the bacterial cells and alleviated the action of detergents, particularly non-ionic and anionic ones, to release recombinant proteins to different extents from the periplasm.

The second category of chemicals used for permeabilisation included chelators (EDTA, DEA, NTA, and SHMP) and chaotropic agents (urea and GndCl), all prepared in 200 mM Tis buffer pH 7.5. Chelating agents can permeabilise the OM by chelating Mg^{2+} and Ca^{2+} crossbridging adjacent LPS molecules and as a result releasing a proportion of LPS from the bacterial cells, replacement of which with phospholipids from the inner leaflet of the OM or from the cytoplasmic membrane creates phospholipid bilayer channels through which hydrophobic compounds can surpass. Weakening the LPS-LPS interaction by the removal of divalent cations may also allow the penetration of small hydrophobic molecules (Nikaido and Vaara, 1985). It has been shown that Tris buffer can synergistically assist the action of chelators (Nikaido and Vaara, 1985; Varra 1992). It has been reported that EDTA (0.3 to 3.0 mM) enhanced excretion of beta lactamase from immobilized *E. coli* cells

and released alkaline phosphatase from the periplasm (Neu and Heppel, 1965; Ryan and Parulekar, 1991). Other chelators, such as HMP and NTA, have been also shown to increase the permeability of the OM and the sensitivity of gram negative bacteria to hydrophobic drugs and detergents (Hancock, 1984; Vaara and Jaakkola, 1989). In the present work, 1 mM EDTA and 10 mM DEA were the most efficient chelators for discharging the periplasmic beta-lactamase and alpha-amylase, and released 44 and 27 per cent beta-lactamase and alpha-amylase from the periplasm, correspondingly (Figs 5.1 and 5.2). As shown in Fig 5.3, EDTA at concentrations of 1 mM and 10 mM discharged the Fab from the periplasm more efficient than osmotic shock (108% and 138%, correspondingly). 10 mM NTA and 0.1% SHMP, releasing respectively 81% and 95% of periplasmic Fab D1.3, were less effectual than EDTA. These chelators caused much less total protein release from the cells when compared to the osmotic shock treatment, and consequently led to much higher specific activity, purification factor and yield factor (Tables 5.2 and 5.3).

Chaotropes, such as urea and GndCl, disorganize the structure of water, by interfering with hydrogen bonds, rendering it less hydrophilic, and weaken solute-solute hydrophobic interactions bringing them into aqueous solution. Chaotropes should usually be employed at high concentration while they are exploited singly for extraction of cytoplasmic proteins (Falconer et al., 1997 and 1999). Nonetheless, it has been evinced that the outer membrane in the presence of low concentrations of chaotropes (e.g. 0.2 mM GndCl) can be disrupted leading to selective protein recovery with high purity from the periplasm (Naglak and Wang, 1990). In recent work, urea at concentrations up to 200 mM released the periplasmic recombinant proteins (31.5% beta-lactamase, and 28% alpha-amylase and Fab D1.3) with a product yield less than that of osmotic shock, although less total protein released by urea caused higher specific activity and purification factor when compared to the osmotic shock treatment. By and large, the efficiency of 200 mM GndCl for permeabilising the cells producing beta-lactamase was the same as urea; however, this concentration of GndCl had no or negligible permeabilisation effect on the strains producing alpha-amylase and Fab D1.3.

The third class of chemicals used for cell permeabilisation in this work included solvents. Solvents probably act by dissolving hydrophobic components, such as the inner and outer membrane of gram negative bacteria (Middelberg, 1995). Solvents have been used for protein recovery from cytoplasm and periplasm of gram negative bacteria. It has been shown that cytoplasmic proteins of *E. coli* are released with a relatively low efficiency by toluene (1-5%), the frequently employed solvent (De Smet *et al*., 1978; Jackson and Demoss, 1965). Chloroform has been employed successfully for the extraction of the biodegradable storage polymer, polyhydroxybutyrate, from various bacteria (Braunegg *et al*., 1978; Lundgren *et al*., 1965). Ames and colleagues (1984) employed different solvents (chloroform, chloroform-toluene, phenethyl alcohol, ether, ether-chloroform and dimethyl sulfoxide) for extraction of periplasmic proteins from gram negative bacteria, and concluded from SDS-PAGE results that chloroform was the most effective solvent leading to total protein recovery and purity comparable to those obtained by osmotic shock. Geckil and colleagues (2004) investigated membrane permeabilisation for the release of periplasmic L-asparaginase from *Enterobacter aerogenes* and *Pseudomonas aeroginosa* using various solvents (hexane, toluene, xylene, benzene and diethylether) made in potassium phosphate buffer, and showed that 1% hexane was the most efficient solvent for permeabilisation in both strains, releasing the periplasmic enzyme with much higher specific activity than that achieved by mechanical disruption (sonication). The higher efficiency of hexane in comparison with other solvents can be attributed to its small size enabling it to penetrate into the outer membrane. In the present work solvents (hexane, xylene, toluene, benzene, diethylether, acetone, chloroform, pyridine, ethanol, and isoamyl alcohol) at 1% concentration were made in 200 mM Tris buffer pH 7.5 and utilized for cell permeabilisation. In terms of priplasmic protein release, in general solvents at 1% concentration were not as effective as osmotic shock treatment. Ten per cent (v/v) isoamyl alcohol was exceptionally effective for periplasmic release of alpha-amylase (75%) and Fab D1.3 (168%), and yielded much higher specific activity, purification factor and yield factor – due to nominal amount of total protein release – when compared to the osmotic shock treatment (Figs 5.1 & 5.2 and Tables 5.2 & 5.3).

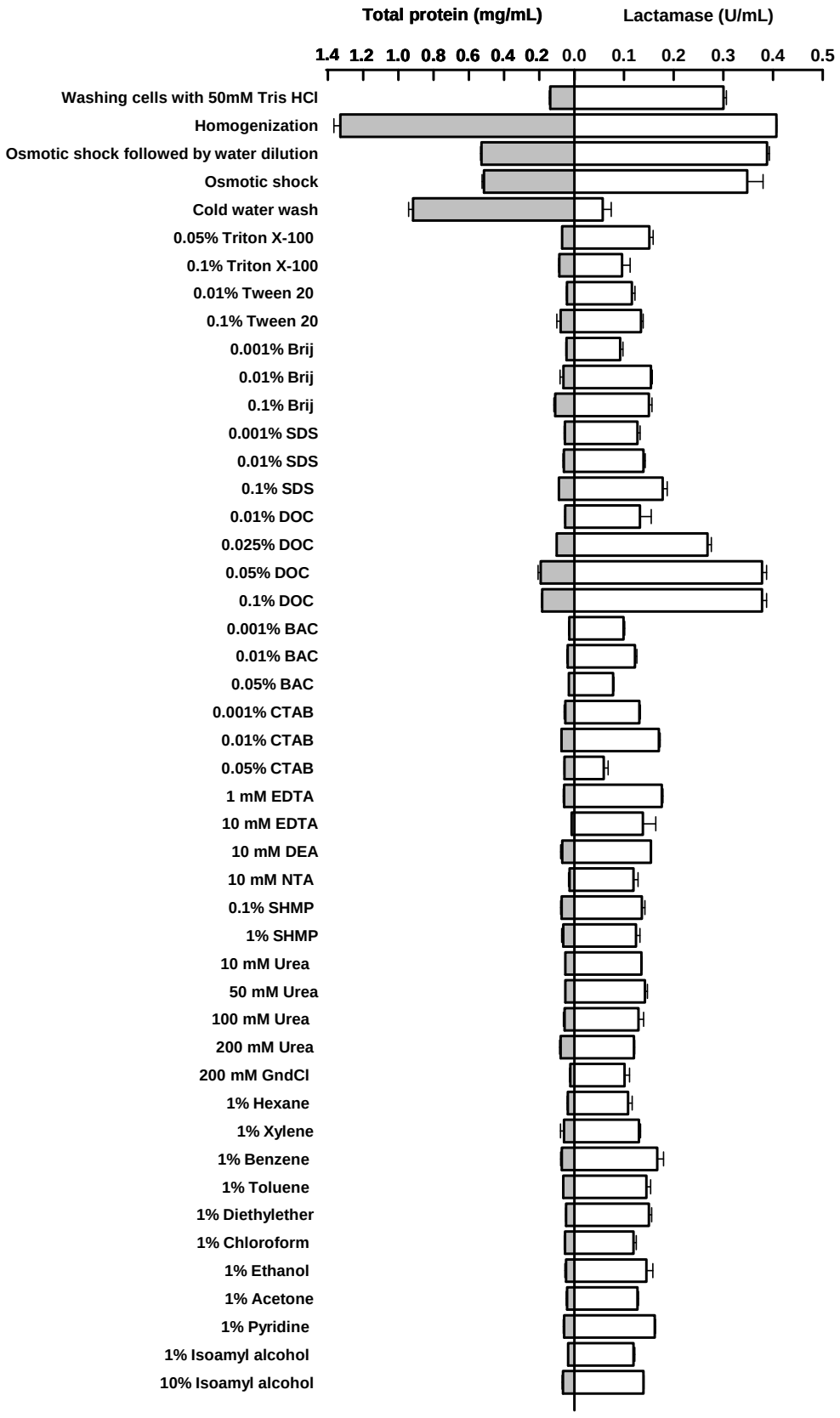

Fig. 5.1 Release of beta-lactamase from transformed *E. coli* JM109 cells by homogenization, osmotic shock and single chemicals made in 200 mM Tris buffer pH 7.5. Results are averages of two measurements.

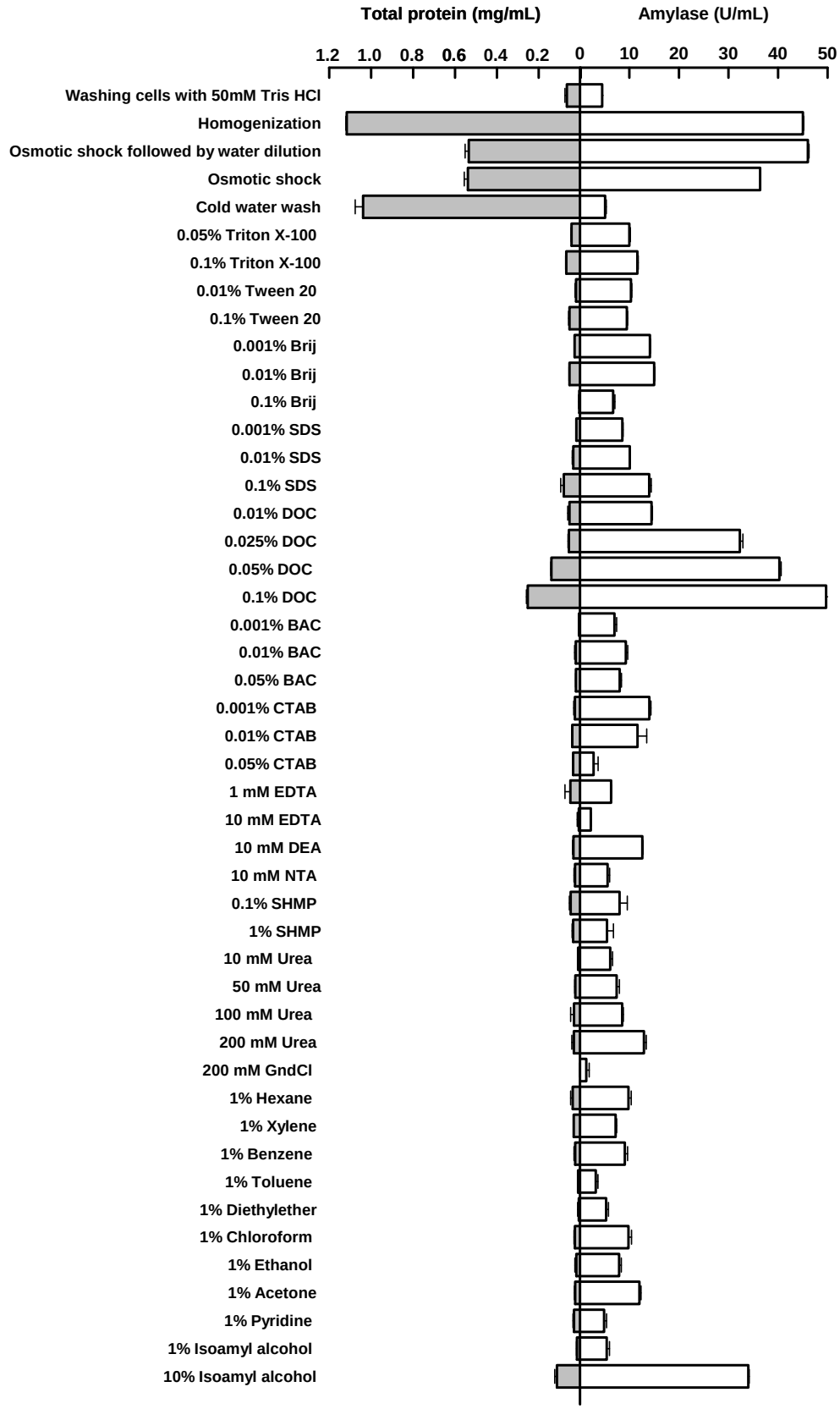

Fig. 5.2 Release of alpha-amylase from *E. coli* JM107 PQR126 cells by homogenization, osmotic shock and single chemicals made in 200 mM Tris buffer pH 7.5. Results are averages of two measurements.

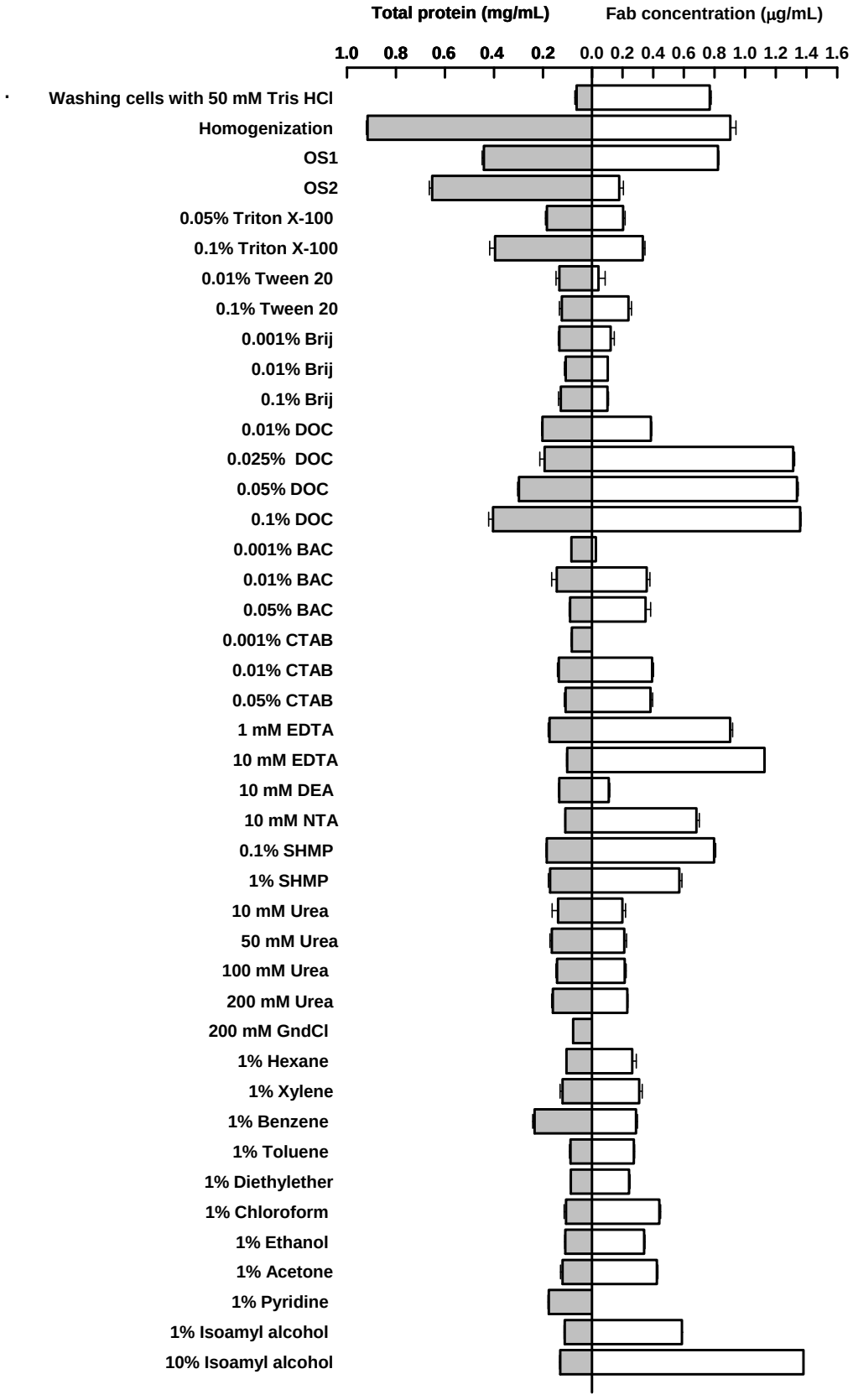

Fig. 5.3 Release of Fab D1.3 from *E. coli* CLD048 cells by homogenization, osmotic shock and single chemicals made in 200 mM Tris buffer pH 7.5. Results are averages of two measurements.

Table 5.2 Comparison between efficiency of homogenization, osmotic shock and various chemicals which release beta-lactamase and alpha-amylase from the periplasm of *E. coli* cells.

Chemicals (prepared in 200mM Tris buffer pH 7.5)	Average recovered enzyme (U/mL/min)		Average Total protein (mg/mL)		Enzyme specific activity (U/mg)		Enzyme yield [a]		Total [b] protein yield		PF [c]		YF% [d]	
	amy. [f]	lac. [g]	amy.	lac.	amy.	lac.	amy.	lac.	amy.	lac.	amy.	lac.	amy.	lac.
Washing cells with 50 mM Tris buffer pH 7.5	**4.48**	**0.30**	**0.07**	**0.14**	**67.98**	**2.18**	**9.73**	**77.37**	**12.34**	**26.11**	**0.79**	**2.96**	**0.08**	**2.29**
Homogenization	**45.03**	**0.41**	**1.12**	**1.33**	**40.34**	**0.31**	**97.85**	**104.90**	**209.06**	**251.87**	**0.47**	**0.42**	**0.46**	**0.44**
Osmotic shock followed by water dilution	**46.02**	**0.39**	**0.53**	**0.53**	**86.20**	**0.74**	**100.00**	**100.00**	**100.00**	**100.00**	**1.00**	**1.00**	**1.00**	**1.00**
Osmotic shock	36.40	0.35	0.54	0.51	67.56	0.68	79.09	89.69	100.89	97.35	0.78	0.92	0.62	0.83
Cold water wash	5.09	0.06	1.04	0.92	4.90	0.06	11.06	14.69	194.52	173.78	0.06	0.08	0.01	0.01
0.05% Triton X-100	9.99	0.15	0.04	0.07	229.97	2.15	21.71	38.92	8.13	13.34	2.67	2.92	0.58	1.14
0.1% Triton X-100	11.58	0.10	0.07	0.09	168.41	1.11	25.16	24.74	12.88	16.32	1.95	1.52	0.49	0.38
0.01% Tween 20	10.30	0.12	0.02	0.04	472.26	2.70	22.38	29.90	4.08	8.13	5.48	3.68	1.23	1.10
0.1% Tween 20	9.47	0.13	0.05	0.08	176.35	1.69	20.58	34.54	10.06	14.98	2.05	2.31	0.42	0.80
0.001% Brij	14.12	0.09	0.03	0.05	487.06	2.02	30.68	23.71	5.43	8.62	5.65	2.75	1.73	0.65
0.01% Brij	14.98	0.15	0.05	0.06	281.95	2.47	32.55	39.69	9.95	11.82	3.27	3.36	1.06	1.33
0.1% Brij	6.68	0.15	0.09	0.11	76.99	1.36	14.52	38.66	16.25	20.84	0.89	1.86	0.13	0.72
0.001% SDS	8.57	0.13	0.02	0.05	415.86	2.31	18.62	32.73	3.86	10.40	4.83	3.15	0.90	1.03
0.01% SDS	10.06	0.14	0.04	0.06	287.18	2.28	21.86	35.82	6.56	11.53	3.33	3.11	0.73	1.11
0.1% SDS	13.99	0.18	0.08	0.09	173.94	2.02	30.40	45.88	15.06	16.70	2.02	2.75	0.61	1.26
0.01% DOC	**14.45**	**0.13**	**0.05**	**0.05**	**269.09**	**2.51**	**31.40**	**34.02**	**10.06**	**9.96**	**3.12**	**3.42**	**0.98**	**1.16**
0.025% DOC	**32.28**	**0.27**	**0.06**	**0.10**	**572.65**	**2.65**	**70.14**	**69.07**	**10.56**	**19.14**	**6.64**	**3.61**	**4.66**	**2.49**
0.05% DOC	**40.27**	**0.38**	**0.14**	**0.19**	**288.14**	**1.97**	**87.51**	**97.42**	**26.17**	**36.41**	**3.34**	**2.68**	**2.93**	**2.61**
0.1% DOC	**49.71**	**0.38**	**0.25**	**0.18**	**196.47**	**2.06**	**108.02**	**97.42**	**47.38**	**34.76**	**2.28**	**2.80**	**2.46**	**2.73**
0.001% BAC	6.96	0.10	0.01	0.03	853.99	3.33	15.12	25.52	1.53	5.63	9.91	4.53	1.50	1.16
0.01% BAC	9.27	0.12	0.02	0.04	389.82	3.14	20.14	31.44	4.45	7.35	4.52	4.28	0.91	1.34
0.05% BAC	8.02	0.08	0.02	0.03	343.32	2.42	17.43	20.10	4.37	6.10	3.98	3.30	0.69	0.66
0.001% CTAB	14.00	0.13	0.03	0.05	507.98	2.51	30.42	33.76	5.16	9.87	5.89	3.42	1.79	1.15
0.01% CTAB	11.65	0.17	0.04	0.07	290.67	2.29	25.32	43.81	7.51	14.07	3.37	3.11	0.85	1.36
0.05% CTAB	2.76	0.06	0.04	0.06	77.38	1.04	6.00	15.21	6.68	10.79	0.90	1.41	0.05	0.21
1 mM EDTA	**6.29**	**0.18**	**0.05**	**0.06**	**126.41**	**2.97**	**13.67**	**45.36**	**9.32**	**11.22**	**1.47**	**4.04**	**0.20**	**1.83**
10 mM EDTA	2.20	0.14	0.01	0.02	260.36	8.42	4.78	35.57	1.58	3.11	3.02	11.45	0.14	4.07
10 mM DEA	**12.60**	**0.15**	**0.03**	**0.07**	**362.28**	**2.22**	**27.38**	**39.69**	**6.51**	**13.17**	**4.20**	**3.01**	**1.15**	**1.20**
10 mM NTA	5.59	0.12	0.03	0.03	208.35	4.30	12.15	30.67	5.02	5.25	2.42	5.85	0.29	1.79
0.1% SHMP	8.00	0.14	0.05	0.07	167.75	1.87	17.38	35.05	8.93	13.75	1.95	2.55	0.34	0.89
1% SHMP	5.46	0.12	0.04	0.06	150.33	1.94	11.86	31.96	6.80	12.14	1.74	2.63	0.21	0.84
10 mM Urea	6.11	0.14	0.01	0.05	485.69	2.61	13.28	34.79	2.36	9.81	5.64	3.55	0.75	1.23
50 mM Urea	7.43	0.14	0.03	0.05	290.69	2.75	16.15	36.60	4.79	9.79	3.37	3.74	0.54	1.37
100 mM Urea	8.50	0.13	0.03	0.06	264.72	2.30	18.47	33.25	6.01	10.61	3.07	3.13	0.57	1.04
200 mM Urea	**12.90**	**0.12**	**0.03**	**0.08**	**391.38**	**1.54**	**28.03**	**30.93**	**6.17**	**14.80**	**4.54**	**2.09**	**1.27**	**0.65**
200 mM GndCl	**1.29**	**0.10**	**0.00**	**0.02**	**586.36**	**4.23**	**2.80**	**26.03**	**0.41**	**4.52**	**6.80**	**5.75**	**0.19**	**1.50**
1% Hexane	9.78	0.11	0.04	0.04	256.36	2.82	21.25	27.84	7.14	7.25	2.97	3.84	0.63	1.07
1% Xylene	7.20	0.13	0.03	0.06	214.48	2.18	15.65	33.51	6.29	11.31	2.49	2.96	0.39	0.99
1% Benzene	9.08	0.17	0.03	0.07	346.83	2.32	19.73	43.04	4.90	13.66	4.02	3.15	0.79	1.36
1% Toluene	3.20	0.15	0.01	0.06	265.56	2.27	6.95	37.37	2.26	12.09	3.08	3.09	0.21	1.16
1% Diethylether	5.29	0.15	0.01	0.05	608.05	3.13	11.50	38.66	1.63	9.09	7.06	4.25	0.81	1.64
1% Chloroform	9.79	0.12	0.03	0.05	352.67	2.20	21.27	30.67	5.20	10.25	4.09	2.99	0.87	0.92
1% Ethanol	7.91	0.15	0.02	0.05	377.21	3.04	17.19	37.37	3.93	9.03	4.38	4.14	0.75	1.55
1% Acetone	11.99	0.13	0.03	0.04	458.33	3.02	26.05	32.73	4.90	7.97	5.32	4.11	1.39	1.34
1% Pyridine	4.88	0.16	0.03	0.06	153.07	2.77	10.60	41.75	5.97	11.10	1.78	3.76	0.19	1.57
1% Isoamyl alcohol	5.42	0.12	0.02	0.04	306.73	3.32	11.78	30.67	3.31	6.79	3.56	4.52	0.42	1.39
10% Isoamyl alcohol	**34.01**	**0.14**	**0.11**	**0.07**	**300.73**	**2.13**	**73.90**	**35.82**	**21.18**	**12.38**	**3.49**	**2.89**	**2.58**	**1.04**

(Continued)

Table 5.2 *Continued*

Chemicals (prepared in 200mM Tris buffer pH 7.5)	Average recovered enzyme (U/mL/min)		Average Total protein (mg/mL)		Enzyme specific activity (U/mg)		Enzyme yield [a]		Total protein yield [b]		PF [c]		YF% [d]	
	amy. [f]	lac. [g]	amy.	lac.	amy.	lac.	amy.	lac.	amy.	lac.	amy.	lac.	amy.	lac.
1 mM EDTA + 0.1% Brij	2.55	0.24	0.08	0.18	31.09	1.31	5.54	60.57	15.36	33.87	0.36	1.79	0.02	1.08
1 mM EDTA + 0.1% Triton X-100	6.29	0.06	0.16	0.22	38.26	0.27	13.67	15.46	30.79	42.29	0.44	0.37	0.06	0.06
1 mM EDTA + 0.1% SDS	7.35	0.22	0.11	0.13	69.03	1.65	15.97	56.70	19.94	25.19	0.80	2.25	0.13	1.28
1 mM EDTA + 0.01% DOC	7.33	0.32	0.07	0.11	108.43	2.94	15.93	82.47	12.66	20.65	1.26	3.99	0.20	3.29
1 mM EDTA + 0.01% CTAB	0.26	0.11	0.01	0.04	26.69	3.07	0.56	28.09	1.82	6.73	0.31	4.17	0.00	1.17
1 mM EDTA + 200 mM Urea	1.32	0.23	0.02	0.08	53.72	2.91	2.87	58.76	4.60	14.84	0.62	3.96	0.02	2.33
1 mM EDTA + 200 mM GndCl	0.00	0.09	0.00	0.02	0.00	4.66	0.00	22.16	0.03	3.50	0.00	6.33	0.00	1.40
1 mM EDTA + 0.01% BAC	0.13	0.10	0.01	0.04	9.04	2.72	0.27	26.80	2.61	7.25	0.10	3.70	0.00	0.99
200 mM Urea + 0.1% Triton X-100	7.41	0.04	0.06	0.12	120.86	0.31	16.10	9.79	11.48	23.39	1.40	0.42	0.23	0.04
200 mM Urea + 0.1% Tween 20	**6.65**	**0.17**	**0.06**	**0.07**	**115.25**	**2.32**	**14.45**	**42.78**	**10.81**	**13.58**	**1.34**	**3.15**	**0.19**	**1.35**
200 mM Urea + 0.1% Brij	**26.12**	**0.05**	**0.12**	**0.14**	**217.56**	**0.38**	**56.76**	**13.14**	**22.48**	**25.67**	**2.52**	**0.51**	**1.43**	**0.07**
200 mM Urea + 0.1% SDS	**34.48**	**0.18**	**0.09**	**0.11**	**396.60**	**1.62**	**74.92**	**46.39**	**16.28**	**20.99**	**4.60**	**2.21**	**3.45**	**1.03**
200 mM Urea + 0.01% DOC	**20.91**	**0.20**	**0.04**	**0.10**	**506.17**	**2.01**	**45.44**	**50.52**	**7.74**	**18.46**	**5.87**	**2.74**	**2.67**	**1.38**
200 mM Urea + 0.025% DOC	**20.82**	**0.10**	**0.05**	**0.11**	**391.87**	**0.90**	**45.24**	**25.77**	**9.95**	**21.05**	**4.55**	**1.22**	**2.06**	**0.32**
200 mM Urea + 0.01% BAC	**7.07**	**0.19**	**0.02**	**0.05**	**285.54**	**3.90**	**15.36**	**48.20**	**4.64**	**9.09**	**3.31**	**5.30**	**0.51**	**2.55**
200 mM Urea + 0.01% CTAB	**39.94**	**0.03**	**0.40**	**0.26**	**98.95**	**0.11**	**86.79**	**7.73**	**75.59**	**49.76**	**1.15**	**0.16**	**1.00**	**0.01**
200 mM GndCl + 0.1% Triton X-100	1.57	0.02	0.04	0.06	43.71	0.38	3.41	5.93	6.73	11.33	0.51	0.52	0.02	0.03
200 mM GndCl + 0.1% Tween 20	0.89	0.09	0.03	0.05	26.06	1.60	1.93	22.42	6.40	10.33	0.30	2.17	0.01	0.49
200 mM GndCl + 0.1% Brij	1.28	0.11	0.04	0.08	31.81	1.29	2.78	27.32	7.54	15.57	0.37	1.75	0.01	0.48
200 mM GndCl + 0.1% SDS	4.17	0.11	0.02	0.03	200.58	3.64	9.06	27.58	3.89	5.57	2.33	4.95	0.21	1.37
200 mM GndCl + 0.01% DOC	2.01	0.13	0.01	0.03	190.88	4.11	4.37	32.47	1.97	5.81	2.21	5.59	0.10	1.82
200 mM GndCl + 0.025% DOC	3.01	0.14	0.02	0.05	151.26	3.01	6.54	36.08	3.73	8.83	1.76	4.09	0.11	1.48
200 mM GndCl + 0.01% BAC	0.96	0.11	0.02	0.05	52.46	2.38	2.09	27.84	3.43	8.59	0.61	3.24	0.01	0.90
200 mM GndCl + 0.01% CTAB	1.63	0.03	0.00	0.02	705.63	1.44	3.54	8.76	0.43	4.49	8.19	1.95	0.29	0.17
1% Hexane + 0.1% Triton X-100	9.70	0.16	0.06	0.07	171.74	2.41	21.08	40.98	10.58	12.50	1.99	3.28	0.42	1.34
1%Hexane + 0.025% DOC	13.90	0.22	0.07	0.07	196.88	3.22	30.20	56.70	13.22	12.96	2.28	4.38	0.69	2.48
1% Hexane + 0.01% CTAB	9.53	0.10	0.02	0.04	411.31	2.70	20.71	26.55	4.34	7.22	4.77	3.68	0.99	0.98
1% Xylene + 0.1% Triton X-100	13.10	0.04	0.05	0.08	250.67	0.50	28.47	10.77	9.79	15.95	2.91	0.68	0.83	0.07
1% Xylene + 0.025% DOC	**41.60**	**0.17**	**0.10**	**0.08**	**431.31**	**2.09**	**90.40**	**42.53**	**18.06**	**14.95**	**5.00**	**2.84**	**4.52**	**1.21**
1% Xylene + 0.01% CTAB	**40.88**	**0.15**	**0.10**	**0.09**	**390.78**	**1.76**	**88.83**	**39.43**	**19.59**	**16.43**	**4.53**	**2.40**	**4.03**	**0.95**
1% Benzene + 0.1% Triton X-100	14.40	0.06	0.07	0.10	201.74	0.54	31.29	14.18	13.37	19.40	2.34	0.73	0.73	0.10
1% Benzene + 0.025% DOC	**44.93**	**0.17**	**0.10**	**0.08**	**428.48**	**2.10**	**97.63**	**42.53**	**19.64**	**14.89**	**4.97**	**2.86**	**4.85**	**1.21**
1% Benzene + 0.01% CTAB	**42.50**	**0.13**	**0.12**	**0.09**	**363.87**	**1.50**	**92.35**	**33.51**	**21.87**	**16.40**	**4.22**	**2.04**	**3.90**	**0.68**
1% Toluene + 0.1% Triton X-100	14.13	0.03	0.06	0.08	242.49	0.43	30.70	8.51	10.91	14.56	2.81	0.58	0.86	0.05
1% Toluene + 0.025% DOC	**28.86**	**0.15**	**0.10**	**0.09**	**294.52**	**1.76**	**62.71**	**39.43**	**18.35**	**16.43**	**3.42**	**2.40**	**2.14**	**0.95**
1% Toluene + 0.01% CTAB	**34.19**	**0.15**	**0.10**	**0.08**	**333.43**	**1.95**	**74.29**	**38.66**	**19.20**	**14.54**	**3.87**	**2.66**	**2.87**	**1.03**
1% Diethyether + 0.1% Triton X-100	10.48	0.17	0.06	0.06	182.36	2.69	22.77	43.30	10.76	11.84	2.12	3.66	0.48	1.58
1% Diethylether + 0.025% DOC	16.70	0.18	0.08	0.08	217.67	2.15	36.29	46.65	14.37	15.92	2.53	2.93	0.92	1.37
1% Diethylether + 0.01% CTAB	13.15	0.11	0.06	0.06	221.79	1.68	28.57	28.09	11.10	12.30	2.57	2.28	0.74	0.64
1% Chloroform + 0.1% Triton X-100	14.39	0.02	0.07	0.06	202.85	0.37	31.27	5.41	13.28	10.64	2.35	0.51	0.74	0.03
1% Chloroform + 0.025% DOC	24.28	0.08	0.07	0.05	334.48	1.58	52.76	20.88	13.59	9.72	3.88	2.15	2.05	0.45
1% Chloroform + 0.01% CTAB	15.39	0.05	0.06	0.04	277.70	1.24	33.44	12.89	10.38	7.61	3.22	1.69	1.08	0.22

(Continued)

Table 5.2 *Continued*

Chemicals (prepared in 200mM Tris buffer pH 7.5)	Average recovered enzyme (U/mL/min)		Average Total protein (mg/mL)		Enzyme specific activity (U/mg)		Enzyme yield [a]		Total protein yield [b]		PF [c]		YF% [d]	
	amy. [f]	lac. [g]	amy.	lac.	amy.	lac.	amy.	lac.	amy.	lac.	amy.	lac.	amy.	lac.
1% Ethanol + 0.1% Triton X-100	12.14	0.18	0.07	0.06	164.61	2.70	26.38	45.10	13.81	12.27	1.91	3.68	0.50	1.66
1% Ethanol + 0.025% DOC	16.70	0.18	0.07	0.09	227.09	1.85	36.29	45.10	13.77	17.95	2.64	2.51	0.96	1.13
1% Ethanol + 0.01% CTAB	28.48	0.18	0.07	0.08	383.00	2.21	61.89	45.62	13.93	15.16	4.44	3.01	2.75	1.37
1% Acetone + 0.1% Triton X-100	12.41	0.16	0.08	0.05	163.61	2.88	26.97	40.46	14.20	10.32	1.90	3.92	0.51	1.59
1% Acetone + 0.025% DOC	17.65	0.18	0.07	0.10	247.03	1.74	38.35	45.88	13.38	19.39	2.87	2.37	1.10	1.09
1% Acetone + 0.01% CTAB	24.53	0.14	0.09	0.06	280.70	2.12	53.30	35.31	16.37	12.26	3.26	2.88	1.74	1.02
1% Pyridine + 0.1% Triton X-100	2.60	0.19	0.55	0.57	4.76	0.33	5.65	48.97	102.28	107.95	0.06	0.45	0.00	0.22
1% Pyridine + 0.025% DOC	18.01	0.26	0.08	0.10	233.32	2.58	39.14	66.24	14.46	18.85	2.71	3.51	1.06	2.33
1% Pyridine + 0.01% CTAB	25.89	0.15	0.08	0.07	317.24	2.25	56.26	38.92	15.28	12.70	3.68	3.06	2.07	1.19
1% Isoamyl alcohol + 0.1% Triton X-100	14.40	0.01	0.09	0.14	164.33	0.06	31.29	2.32	16.41	26.50	1.91	0.09	0.60	0.00
1% Isoamyl alcohol + 0.025% DOC	18.31	0.19	0.06	0.08	322.02	2.45	39.79	47.94	10.65	14.39	3.74	3.33	1.49	1.60
1% Isoamyl alcohol + 0.01% CTAB	17.55	0.14	0.07	0.06	235.82	2.19	38.14	35.82	13.94	12.02	2.74	2.98	1.04	1.07

[a] and [b] Relative to extraction by osmotic shock followed by water dilution

[c] Enzyme yield divided to total protein yield

[d] Enzyme yield multiply by PF divided to 100

[f] Strain producing alpha-amylase

[g] Strain producing beta-lactamase

Table 5.3 Comparison between efficiency of homogenization, osmotic shock and various chemicals which release Fab D1.3 from the periplasm of *E. coli* cells.

Chemicals (prepared in 200 mM Tris beffer pH 7.5	Average D1.3 concentration (mg/mL)	Average Total protein (mg/mL)	D1.3 yield [a]	Total protein yield [b]	PF [c]	YF% [d]
Washing cells with 50 mM Tris HCl	**0.77**	**62.7**	**93.67**	**14.22**	**6.59**	**6.17**
Homogenisation	**0.90**	**916.39**	**109.73**	**207.87**	**0.53**	**0.58**
OS1	**0.82**	**440.85**	**100.00**	**100.00**	**1.00**	**1.00**
OS2	0.18	652.35	21.65	147.98	0.15	0.03
0.05% Triton X-100	0.20	184.18	24.70	41.78	0.59	0.15
0.1% Triton X-100	0.33	395.78	40.39	89.78	0.45	0.18
0.01% Tween 20	0.04	132.93	5.23	30.15	0.17	0.01
0.1% Tween 20	0.24	123.03	28.95	27.91	1.04	0.30
0.001% Brij	0.12	133.50	14.84	30.28	0.49	0.07
0.01% Brij	0.10	106.97	12.53	24.26	0.52	0.06
0.1% Brij	0.03	127.21	3.28	28.86	0.11	0.00
0.01% DOC	0.38	202.38	46.23	45.91	1.01	0.47
0.025% DOC	**1.31**	**193.35**	**159.73**	**43.86**	**3.64**	**5.82**
0.05% DOC	**1.34**	**298.41**	**162.65**	**67.69**	**2.40**	**3.91**
0.1% DOC	**1.36**	**403.81**	**165.33**	**91.60**	**1.80**	**2.98**
0.001% BAC	0.03	84.12	3.04	19.08	0.16	0.00
0.01% BAC	0.35	143.59	42.58	32.57	1.31	0.56
0.05% BAC	0.08	89.09	9.73	20.21	0.48	0.05
0.001% CTAB	0.00	82.07	0.00	18.62	0.00	0.00
0.01% CTAB	0.39	135.39	47.69	30.71	1.55	0.74
0.05% CTAB	0.09	107.75	10.34	24.44	0.42	0.04
1 mM EDTA	**0.90**	**173.78**	**109.49**	**39.42**	**2.78**	**3.04**
10 mM EDTA	**1.13**	**101.52**	**136.98**	**23.03**	**5.95**	**8.15**
10 mM DEA	0.11	133.77	13.26	30.34	0.44	0.06
10 mM NTA	**0.68**	**109.05**	**82.97**	**24.74**	**3.35**	**2.78**
0.1% SHMP	**0.79**	**184.77**	**96.11**	**41.91**	**2.29**	**2.20**
1% SHMP	0.57	171.26	69.34	38.85	1.78	1.24
10 mM Urea	0.20	138.41	24.09	31.40	0.77	0.18
50 mM Urea	0.21	163.94	25.67	37.19	0.69	0.18
100 mM Urea	0.21	142.60	26.03	32.35	0.80	0.21
200 mM Urea	0.23	160.04	28.10	36.30	0.77	0.22
200 mM GndCl	0.00	76.78	0.00	17.42	0.00	0.00
1% Hexane	0.26	104.62	32.00	23.73	1.35	0.43
1% Xylene	0.31	120.34	37.47	27.30	1.37	0.51
1% Benzene	0.29	234.39	34.91	53.17	0.66	0.23
1% Toluene	0.27	87.84	33.21	19.93	1.67	0.55
1% Diethylether	0.24	86.55	29.44	19.63	1.50	0.44
1% Chloroform	0.44	106.11	53.41	24.07	2.22	1.19
1% Ethanol	0.34	109.00	41.36	24.72	1.67	0.69
1% Acetone	0.42	120.42	51.58	27.32	1.89	0.97
1% pyridine	0.00	176.09	0.00	39.94	0.00	0.00
1% Isoamyl alcohol	0.59	111.05	71.17	25.19	2.83	2.01
10% Isoamyl alcohol	1.38	130.04	167.88	29.50	5.69	9.55

(Continued)

Table 5.3 *Continued*

Chemicals (prepared in 200 mM Tris beffer pH 7.5	Average D1.3 concentration (mg/mL)	Average Total protein (mg/mL)	D1.3 yield [a]	Total protein yield [b]	PF [c]	YF% [d]
1 mM EDTA + 0.1% Brij	**1.12**	**246.02**	**136.50**	**55.81**	**2.45**	**3.34**
1 mM EDTA + 0.1% Triton X-100	**1.20**	**396.29**	**145.99**	**89.89**	**1.62**	**2.37**
1 mM EDTA + 0.01% DOC	**1.11**	**230.17**	**135.04**	**52.21**	**2.59**	**3.49**
1 mM EDTA + 0.01% CTAB	**1.11**	**163.29**	**135.04**	**37.04**	**3.65**	**4.92**
1 mM EDTA + 200 mM Urea	**0.94**	**203.43**	**113.75**	**46.14**	**2.46**	**2.80**
1 mM EDTA + 200 mM GndCl	0.00	89.62	0.00	20.33	0.00	0.00
1 mM EDTA + 0.01% BAC	**0.89**	**172.63**	**107.91**	**39.16**	**2.76**	**2.97**
200 mM Urea + 0.1% Triton X-100	0.41	259.47	49.64	58.86	0.84	0.42
200 mM Urea + 0.1% Tween 20	0.00	140.74	0.00	31.92	0.00	0.00
200 mM Urea + 0.1% Brij	0.06	152.24	7.30	34.53	0.21	0.02
200 mM Urea + 0.01% DOC	0.08	129.52	9.73	29.38	0.33	0.03
200 mM Urea + 0.025% DOC	0.06	123.58	7.30	28.03	0.26	0.02
200 mM Urea + 0.01% BAC	0.25	132.81	30.41	30.13	1.01	0.31
200 mM Urea + 0.01% CTAB	0.00	350.72	0.00	79.56	0.00	0.00
200 mM GndCl + 0.1% Triton X-100	0.00	152.05	0.00	34.49	0.00	0.00
200 mM GndCl + 0.1% Tween 20	0.00	101.28	0.00	22.97	0.00	0.00
200 mM GndCl + 0.1% Brij	0.00	116.63	0.00	26.46	0.00	0.00
200 mM GndCl + 0.01% DOC	0.00	73.60	0.00	16.70	0.00	0.00
200 mM GndCl + 0.025% DOC	0.00	97.20	0.00	22.05	0.00	0.00
200 mM GndCl + 0.01% BAC	0.00	99.07	0.00	22.47	0.00	0.00
200 mM GndCl + 0.01% CTAB	0.00	58.09	0.00	13.18	0.00	0.00
1% Hexane + 0.1% Triton X-100	**0.82**	**133.68**	**99.64**	**30.32**	**3.29**	**3.27**
1% Hexane + 0.025% DOC	**1.33**	**134.42**	**161.56**	**30.49**	**5.30**	**8.56**
1% Hexane + 0.01% CTAB	0.57	139.66	69.59	31.68	2.20	1.53
1% Xylene + 0.1% Triton X-100	**0.86**	**310.16**	**104.74**	**70.35**	**1.49**	**1.56**
1% Xylene + 0.025% DOC	0.27	137.29	32.73	31.14	1.05	0.34
1% Xylene + 0.01% CTAB	0.51	141.30	62.53	32.05	1.95	1.22
1% Benzene + 0.1% Triton X-100	1.35	301.09	164.48	68.30	2.41	3.96
1% Benzene + 0.025% DOC	0.31	146.64	38.08	33.26	1.14	0.44
1% Benzene + 0.01% CTAB	0.32	156.66	39.29	35.54	1.11	0.43
1% Toluene + 0.1% Triton X-100	**0.87**	**216.71**	**105.84**	**49.16**	**2.15**	**2.28**
1% Toluene + 0.025% DOC	0.21	147.90	25.79	33.55	0.77	0.20
1% Toluene + 0.01% CTAB	0.00	144.55	0.00	32.79	0.00	0.00
1% Diethyether + 0.1% Triton X-100	0.69	132.35	84.06	30.02	2.80	2.35
1% Diethylether + 0.025% DOC	0.19	159.04	22.87	36.08	0.63	0.14
1% Diethylether + 0.01% CTAB	0.43	156.81	52.19	35.57	1.47	0.77
1% Chloroform + 0.1% Triton X-100	0.64	187.42	77.37	42.51	1.82	1.41
1% Chloroform + 0.025% DOC	0.59	174.71	71.29	39.63	1.80	1.28
1% Chloroform + 0.01% CTAB	0.34	154.33	41.85	35.01	1.20	0.50

(Continued)

Table 5.3 *Continued*

Chemicals (prepared in 200 mM Tris beffer pH 7.5	Average D1.3 concentration (mg/mL)	Average Total protein (mg/mL)	D1.3 yield [a]	Total [b] protein yield	PF [c]	YF% [d]
1% Ethanol + 0.1% Triton X-100	0.62	142.16	74.94	32.25	2.32	1.74
1% Ethanol + 0.025% DOC	0.48	165.73	57.91	37.59	1.54	0.89
1% Ethanol + 0.01% CTAB	0.47	160.93	57.54	36.50	1.58	0.91
1% Acetone + 0.1% Triton X-100	0.66	141.64	79.93	32.13	2.49	1.99
1% Acetone + 0.025% DOC	0.16	162.81	19.83	36.93	0.54	0.11
1% Acetone + 0.01% CTAB	0.46	151.30	56.33	34.32	1.64	0.92
1% Pyridine + 0.1% Triton X-100	0.00	826.65	0.00	187.51	0.00	0.00
1% Pyridine + 0.025% DOC	0.00	163.63	0.00	37.12	0.00	0.00
1% Pyridine + 0.01% CTAB	0.00	139.01	0.00	31.53	0.00	0.00
1% Isoamyl alcohol + 0.1% Triton X-100	0.80	242.34	96.96	54.97	1.76	1.71
1% Isoamyl alcohol + 0.025% DOC	0.12	137.97	14.36	31.30	0.46	0.07
1% Isoamyl alcohol + 0.01% CTAB	**0.74**	**173.26**	**90.51**	**39.30**	**2.30**	**2.08**

[a] and [b] Relative to extraction by osmotic shock solution 1

[c] Fab D1.3 yield divided to total protein yield

[d] Fab D1.3 yield multiply by PF divided to 100

In the present work, the influences of different combinations of chemicals on protein release from the periplasm were also investigated. Detergents can be employed at low concentration and in combination with other treatments (e.g., EDTA and chaotropes) for destabilizing the OM of gram-negative bacteria and cell disrupting (Hettwer and Wang, 1989; Middelberg, 1995). The LPS layer in gram negative bacteria is deemed to protect the phospholipids in the inner leaflet of the OM and in the inner (cytoplasmic) membrane from destructive agents such as neutral and anionic detergents. EDTA chelates Mg^{2+} and Ca^{2+} crossbridging adjacent LPS and releases a proportion of LPS from the bacterial cells. The released LPS can be replaced with phospholipids from the inner leaflet of the OM or from the cytoplasmic membrane creating phospholipid bilayer channels which detergents can act on. In the present work, EDTA and detergents/chaotropes were combined and utilised for cell permeabilisation to extract periplasmic proteins from *E. coli* cells. EDTA (1 mM) did not increase the efficiency of detergents and chaotropes to release alpha-

amylase; however; 1 mM EDTA enhanced to some extent the beta-lactamase release efficacy of 0.1% Brij, 0.1% SDS, 0.01% DOC, and 200 mM urea (Figs 5.4 and 5.5). Combination of 1 mM EDTA with 0.1% Brij, 0.1% Triton X-100, 0.01% DOC, 0.01% CTAB, 0.01% BAC, and 200 mM urea could also release Fab D1.3 considerably higher than either of single chemicals (Fig 5.6). These combinations of chemicals, which had synergetic effects, discharged a smaller amount of total protein from the cells when compared to osmotic shock treatment, and as a result led to higher enzyme specific activity, purification factor and yield factor (Tables 5.2 and 5.3). Novella and colleagues (1994) showed that 95% of penicillin acylase with a purification factor of 25, when compared to disruption by sonication, was extracted from the periplasm of *E. coli* by combined use of 10 mM EDTA and 10 mM GndCl at room temperature for 10 h. However in the present study, the amount of periplasmic release by combination of 1 mM EDTA and 200 mM GndCl was less than 1 mM EDTA alone. The reason for this antagonism is not known.

The effect of chaotropes on release efficiency of cationic, anionic and non-anionic detergents was also studied. Urea (200 mM) partly increased the efficiency of 0.1% Tween, 0.1% SDS, 0.01% DOC and 0.01% BAC for releasing beta-lactamase (Fig 5.4). It has been shown that the combination of Triton X-100 (0.5%) and GndCl (100 mM) can results in significant amount of protein release from the cytoplasm of *E. coli*, while these are individually incapable of inducing a noticeable amount of protein release (Hettwer and Wang, 1989); however in the recent study, GndCl (200 mM) had negative impact on the efficiency of the detergents to release beta-lactamase. Urea (200 mM) also enhanced the alpha-amylase release effectiveness of 0.1% Brij, 0.1% SDS, 0.01% DOC, and 0.01% CTAB, while 200 mM GndCl had negative effect on the efficiency of the detergents to release alpha-amylase (Fig 5.5). The reason for the antagonism between 200 mM GndCl and the detergents is not known. The maximum synergetic effects were attributed to the combination of 200 mM urea/0.1% SDS and 200 mM urea/0.01% CTAB which discharged approximately 90 and 100 per cent of periplasmic alpha-amylase, respectively. Urea and GndCl (200 mM) had no positive effects on the performance of detergents to extract Fab D1.3 from the cells (Fig 5.6).

The combinations of solvents (at 1% concentration) and detergents were finally used to evaluate their performances for release of periplasmic proteins. While the combination of solvents and detergents did not increase beta-lactamase release figures attributable to either single chemicals, such combinations could augment the release of alpha-amylase from the cells. For example, combinations of xylene, benzene, and toluene (at 1% concentration) with 0.025% DOC and 0.01 % CTAB considerably enhanced alpha-amylase discharge (up to 100%) from the periplasm (Fig 5.5). Total protein release by such combinations was much less than osmotic shock, and as a result higher enzyme specific activity, purification factor and yield factor were obtained (Table 5.2). Also, such chemical combinations can be more effective than single chemicals in releasing Fab D1.3 from the periplasm; for instance, combining hexane, xylene, toluene and isoamyl alcohol at 1% concentration with 0.1% Triton X-100 could release the periplasmic Fab as efficiently as osmotic shock and greater than either single chemical (Fig 5.6, and Table 5.3).

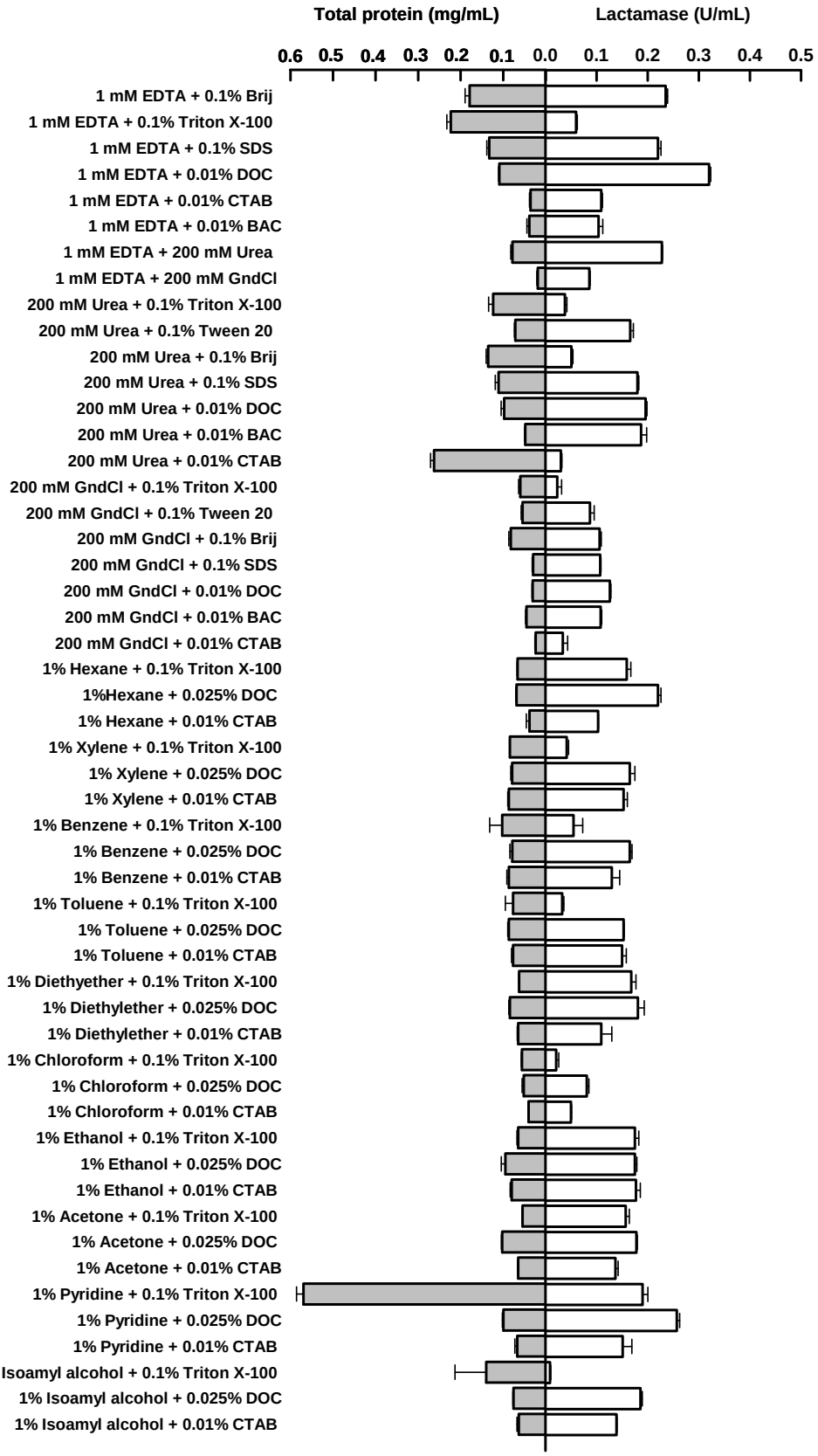

Fig. 5.4 Release of beta-lactamase from transformed *E. coli* JM109 cells by combinations of chemicals made in 200 mM Tris buffer pH 7.5. Results are averages of two measurements.

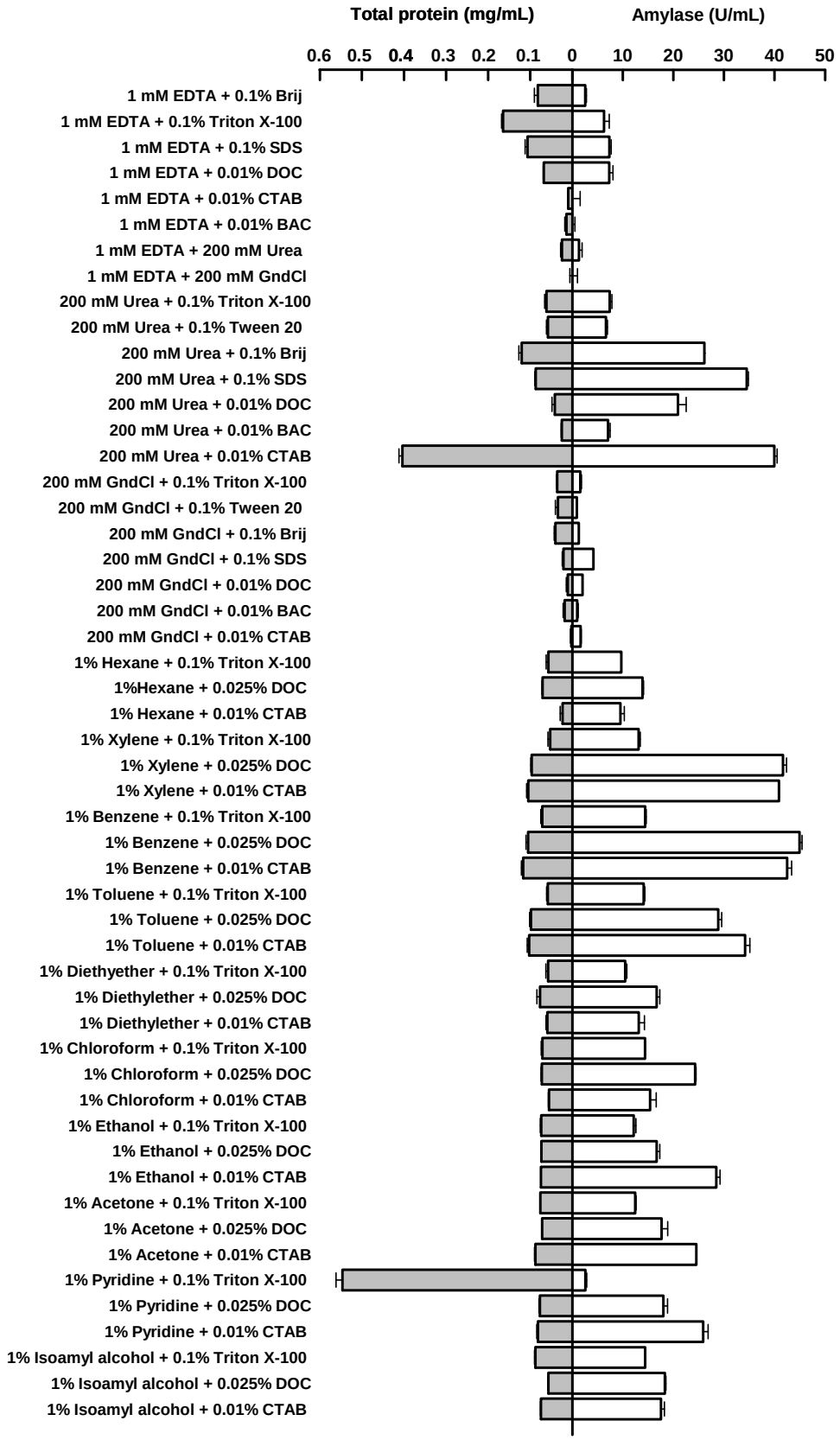

Fig. 5.5 Release of alpha-amylase from *E. coli* JM107 PQR126 cells by combinations of chemicals made in 200 mM Tris buffer pH 7.5. Results are averages of two measurements.

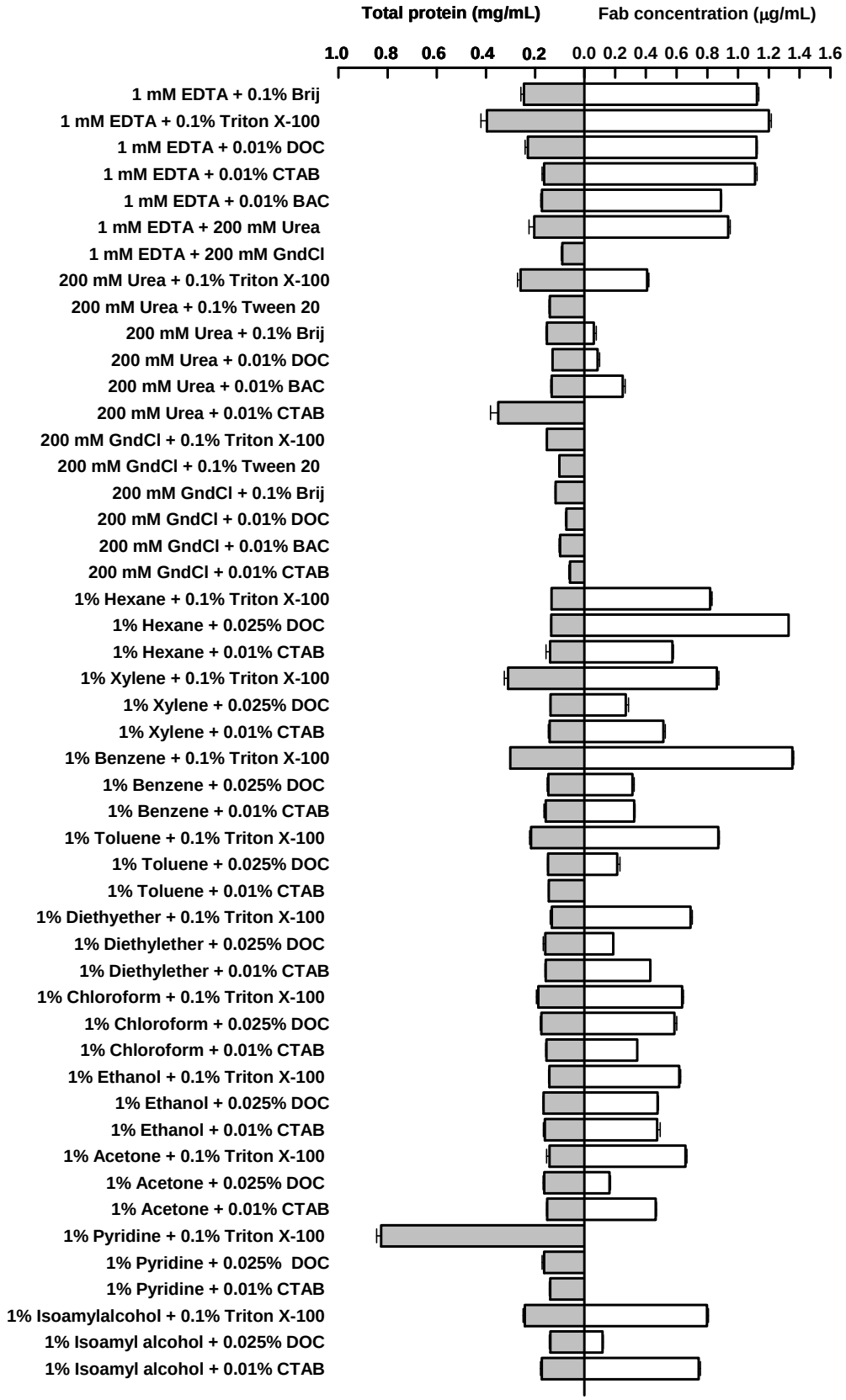

Fig. 5.6 Release of Fab D1.3 from *E. coli* CLD048 cells by combinations of chemicals made in 200 mM Tris buffer pH 7.5. Results are averages of two measurements.

5.4 Conclusions

Prior to commencing cell permeabilization experiments, the influences of different concentrations of various chemicals, having been recognized as potential bacterial cell permeabilisers, on the structure and biological activity of three model proteins were investigated in Chapter 4, and the results obtained were used in this chapter to design and evaluate extraction procedures for the three test proteins (i.e. beta-lactamase, alpha-amylase and Fab D1.3) from the periplasm of recombinant *E. coli*. It was shown that washing cells with 50 mM Tris buffer pH 7.5 could release recombinant protein from the periplasm; for instance, 43% and 48% of the periplasmic beta-lactamase and Fab D1.3, respectively, were discharged by this step.

The efficiency of classic and modified osmotic shock procedures for releasing periplasmic beta-lactamase and alpha-amylase was evaluated and it was shown that the modified osmotic shock procedure was more efficient than the classic one and as efficient as high pressure homogenization. The higher efficiency of the modified osmotic shock has been also demonstrated by other researchers (French *et al*., 1996).

It was not possible to include HEWL in the osmotic shock fluid used for periplasmic extraction of Fab D1.3, because HEWL is the specific antigen for the Fab and its strong attachment to the antibody fragment interferes with the Fab D1.3 analysis, which is based on the attachment of Fab D1.3 to the immobilised HEWL on microplate. However, osmotic shock solution lacking HEWL was almost as effective as high pressure homogenization for periplasmic release of Fab D1.3.

The safe chemicals, not having detrimental impacts on the biological activity of the target proteins, were applied at low concentrations – singly and in various combinations – for protein release from the periplasm of recombinant *E. coli*. Highly selective high yielding chemical extraction treatments were identified in all three cases. Amongst single chemicals, sodium DOC at concentrations ranged from 0.025% to 0.1% seems to be a general efficient permeabiliser, and is capable to

extract the three target proteins even more effectual than osmotic shock disruption. The amount of total protein release by DOC at the mentioned concentrations was less than that by osmotic shock, and as a result DOC treatment resulted in higher specific activity, purification factor and yield factor. These higher values improve the yield of recombinant protein purification in downstream processing. Apart from DOC, the influence of the exposure to other chemicals is product dependent. For example, ten per cent (v/v) isoamyl alcohol is able to effectively extract periplasmic alpha-amylase (75%) and Fab D1.3 (168%), but its performance for beta-lactamase extraction from the periplasm is low (26%). Also chelating agents such as 1-10 mM EDTA, 10 mM NTA, and 0.1-1% SHMP only released Fab D1.3 from the periplasm very efficiently, while the release of beta-lactamase and alpha-amylase by chelating agents was much less efficient than osmotic shock.

Certain chemicals showed synergetic effects on each other; for example, 1 mM EDTA can increase Fab D1.3 and/or beta-lactamase release when combined with detergents such as 0.1% Brij, 0.1% SDS, 0.1% Triton X-100, 0.01% DOC, 0.01% CTAB, 0.01% BAC and 200 mM urea. Also detergents, such as 0.1% Tween, 0.1% Brij, 0.1% SDS, 0.01% DOC, 0.01% CTAB and 0.01% BAC could release greater beta-lactamase and/or alpha-amylase when combined with 200 mM urea. One per cent solvents (e.g. hexane, xylene, benzene, toluene and isoamyl alcohol) showed marked capability for releasing alpha-amylase and and/or Fab D1.3 when they are used in combination with detergents such as 0.025% DOC, 0.01 % CTAB and 0.1% Triton X-100. These combinations of chemicals caused higher specific activity, purification factor and yield factor.

The low concentrations of single and combined chemicals can be considered as alternatives to osmotic shock disruption for selective extraction of periplasmically-expressed recombinant proteins. These can be superior selective extraction techniques at large scale, where the classic osmotic shock can not be used, because of being expensive and unfeasible.

5.5 References

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6 Concluding remarks

Escherichia coli has been used for many years for manufacturing many recombinant proteins. When secretory proteins containing disulphide bonds in the final structure are produced without signal peptides, they remain in the cytoplasm and can aggregate and form inclusion bodies. This is undesirable outcome particularly if the protein contains multiple non-linear disulphide bonds and consequently difficult to solubilise and refold into its active conformation. Therefore in recent years, many industrially important recombinant proteins, which contain disulphide bonds in their structure, have been directed into the periplasm of *E. coli*, inasmuch as the periplasm provides the conditions required for appropriate disulphide bond formation and protein folding. The quantity of soluble periplasmic recombinant protein can be maximized by manipulation of the solute composition of the periplasm, via adjusting the culture medium composition, and/or by genetic manipulation of *E. coli* (Chapter 1).

In spite of the advances in high level production of recombinant proteins in the periplasm, periplasmic expression systems have not yet fulfilled their potential, due to the lack of reliable general methods for proficient selective release of periplasmically expressed proteins at large-scale. Classical osmotic shock is the only reliable technique for selective extraction of periplasmic proteins; however, this technique is labour intense and can only be used on small volumes. Thus, developing new methods, being able to selectively release the periplasmic proteins and able to scale up, is urgently required. Therefore, the motivation for the works presented in this thesis has been to develop new ‘gentle’ chemical methods for selective release of recombinant proteins from the periplasm of *E. coli*. The *E. coli* strains used in this work produced beta-lactamase, alpha-amylase, and Fab D1.3 in the periplasm.

Prior to developing chemical methods for periplasmic extraction of recombinant proteins, the influence of such chemicals on the structure and biological activity of

such target proteins ought to be investigated. Therefore, pure proteins were required for such investigations.

A common characteristic of many *E. coli* secretion systems is that the product may appear in many various locations within and outside the cells In Chapter 2, fed-batch cultivation was conducted at 37°C for production of Fab D1.3 in a recombinant *E. coli*. It was demonstrated that Fab D1.3 produced in the periplasm and then secreted into the culture medium. The variations in the titre of the soluble Fab in different locations (i.e. the cytoplasm, the periplasm, and the culture medium) over the course of fed-batch cultivation were determined. It was shown that Fab D1.3 was largely secreted into the culture medium after 4 h induction with 0.1 mM IPTG.

Based on the results obtained in Chapter 2, further chromatographic purification works were carried out to purify Fab D1.3 from the culture broth (Chapter 3). By using coupled HiTrap SP Sepharose XL and Protein G columns, highly purified (>97%) Fab D1.3 was achieved. It was shown that the performance of coupled HiTrap SP Sepharose XL and Protein G columns was much higher than the performance of coupled HEWL and Protein G affinity columns.

The purified Fab D1.3 by coupled HiTrap SP Sepharose XL and Protein G columns and pure beta-lactamase and alpha-amylase purchased from Sigma-Aldrich (Gillingham, UK) were used for structural and biological activity studies in the presence of a numerous recognised cell permeabilisation reagents (Chapter 4). The alterations in the secondary structure of the proteins, when exposed to different chemicals, were investigated by a newly developed high throughput circular dichroism (ht-CD). Ht-CD could use for detecting changes in the secondary structure of the proteins occurred by chemicals, for instance by measuring the intensity of the CD spectra at key wavelengths (i.e. 222 nm for beta-lactamase and alpha-amylase, and 214 nm for Fab D1.3). However, the changes in the secondary structure of proteins were compared with the changes in the biological activity of the proteins in the presence of various chemicals. This comparison, demonstrated that some chemicals might have deleterious impact on biological activity of the proteins and subtly alter the structure of proteins in way that are not recorded by ht-CD. Also,

some chemicals might change the secondary structures protein, which could be recorded by ht-CD, but might not cause any reduction in the biological activity of the proteins; for example, because such structural changes happened outside the active site of proteins or occurred in a manner that the biological activity remained intact.

Certain chemicals, such as chelating agents, surfactants, chaotropes, and solvents, have been recognised to induce protein release by altering the cell envelope structure and allowing diffusion of the product from gram-negative bacteria. Many raucous chemical methods have been developed to extract intracellular proteins from *E. coli* by permeabilising outer wall barriers. However, very few attempts have been made for developing gentle and selective chemical methods to extract recombinant proteins from the periplasm of *E. coli*. Chemicals which did not influence the biological activity of the proteins (Chapter 4) were utilised – singly and in various combinations – for permeabilisation of *E. coli* cells harbouring the same target species (Chapter 5). Amongst chemicals, which were singly used for extraction of proteins from the periplasm of *E. coli*, sodium deoxycholate (DOC) at low concentrations ranged from 0.025% to 0.1% was shown to be a general permeabiliser because it was able to release all three target periplasmic proteins even more efficient than osmotic shock. The influence of exposure to certain single chemicals was obviously product dependent; for instance, 10% isoamyl alcohol was exceptionally effective for periplasmic release of alpha-amylase (75%) and Fab D1.3 (168%). It was shown that when certain chemicals used together at low concentrations, they exerted synergistic effects. However, the effects of exposure to combinations of chemicals varied from protein to protein (see Figs 5.4 to 5.6, and Tables 5.2 and 5.3 presented in Chapter 5). It was demonstrated in this thesis that selective extraction of periplasmic proteins, with releasing only small amount of total cell protein, from *E. coli* could be achieved. The permeabilised cells are not fragmented and the viscosity of the treatment solution is low; thus, the permeabilised cells can easily separated by centrifugation. This avoidance of extensive clarification steps can help out subsequent recovery and purification of periplasmic proteins in a downstream process.

Clearly, future application of the developed chemical cocktails in this thesis is of interest. Effect of chemicals additives on the tertiary structure of these target proteins may need to be investigated by measuring CD spectra in near-UV region using this ht-CD system. In the present work, the Michaelis-Menten constant (K_M) of enzymes in the presence of different chemicals was not determined; therefore, for each chemical the enzymes reaction rates may need to be measured in the presence of various concentrations of the substrates in order to measure values of K_M. Further studies are to be done to demonstrate exploitation of the developed chemical permeabilisers in this thesis for selective release of other industrially relevant proteins (such as various antibody fragments, etc.) from the periplasm of recombinant *E. coli*. The influence of upstream changes (i.e. media composition, point of induction, phase of growth) on the optimal release is to be investigated. Given the marked changes that can occur in composition of cell envelopes under different growth regimes it will be interesting to see whether the make-up of the chemical cocktail used to permeabilise the cells needs adjusting.

7 Appendix

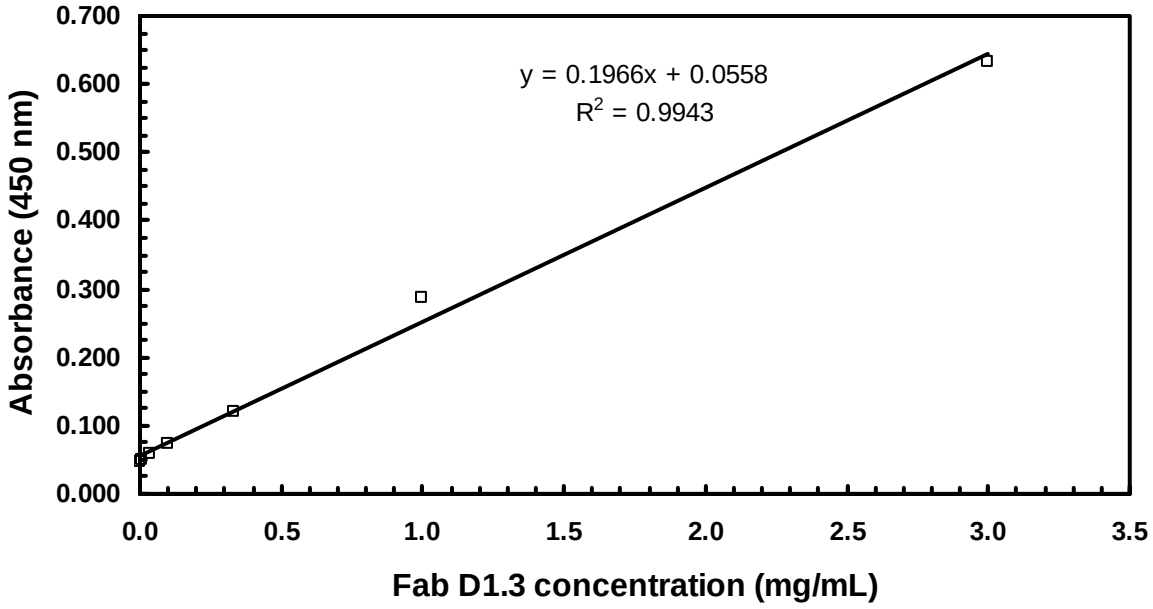


Fig. 7.1 Calibration curve used for calculation of Fab D1.3 concentration in the samples by ELISA.

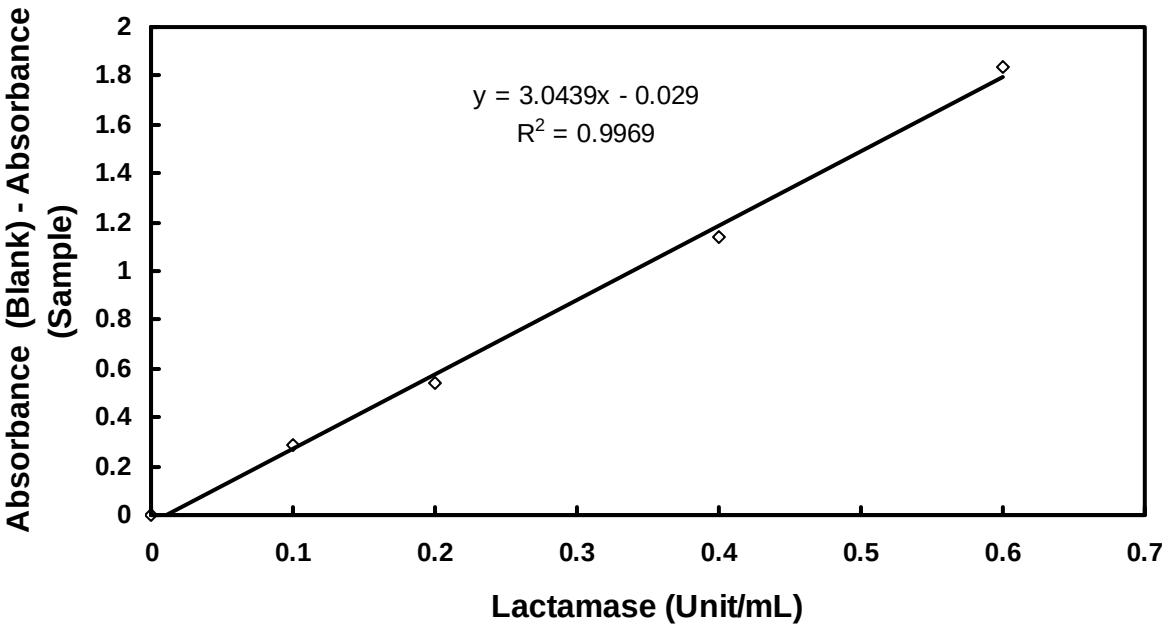


Fig. 7.2 Calibration curve used for calculation of beta-lactamase concentration via absorbance measurements at 450 nm.

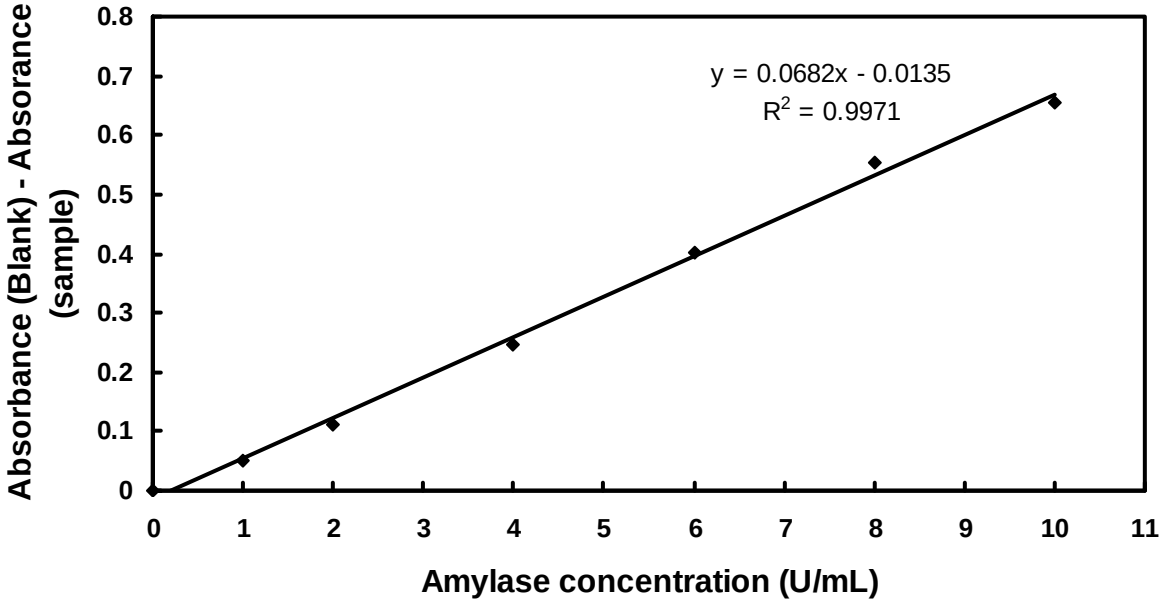


Fig. 7.3 Calibration curve used for calculation of alpha-amylase concentration via absorbance measurements at 600 nm.

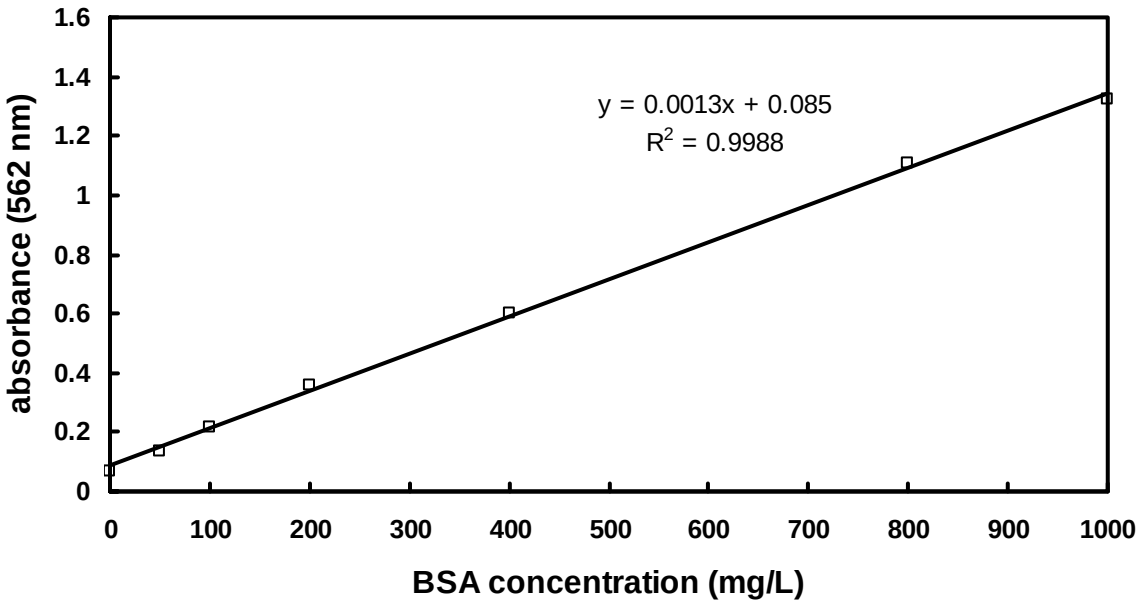


Fig. 7.4 Calibration curve used for calculation of total cell protein via absorbance measurements at 562 nm.

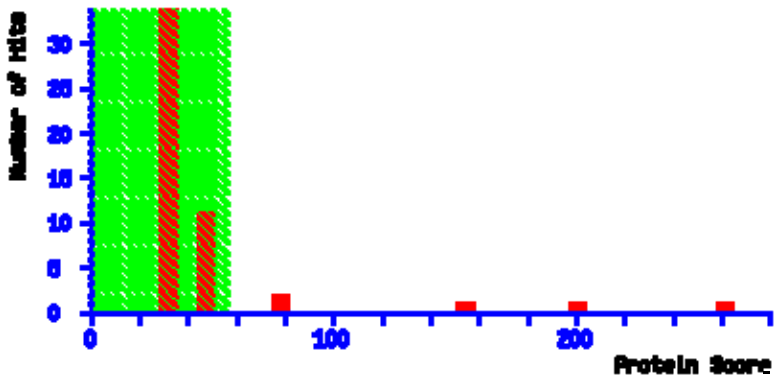
Table 7.1 Mascot search results for the mass spectrometry analysis of the top band of lane 6 in figure 3.6.

User : Jonathan James
Email : j.d.james.1@bham.ac.uk
Search title :
MS data file : C:\Documents and Settings\jamesjdz\Desktop\RJ_260808.pkl
Database : MSDB 20060831 (3239079 sequences; 1079594700 residues)
Timestamp : 29 Aug 2008 at 09:49:18 GMT

Protein hits :		
	E1007993	SYNTHETIC PUC19 FABD1.3 SEQUENCE.- vectors.
	Q6PI81_HUMAN	IGHM protein.- Homo sapiens (Human).
	AAS85976	AY393038 NID: - Homo sapiens
	Q4CR60_TRYCR	Trans-sialidase, putative (Fragment).- Trypanosoma cruzi.
	PH1422	Ig heavy chain V region (clone P3-69) - human (fragment)
	TRPGTR	trypsin (EC 3.4.21.4) precursor - pig (tentative sequence)
	Q5CJY7_CRYHO	Hypothetical protein.- Cryptosporidium hominis.
	Q2QUE5_ORYSA	Wall-associated kinase-like 1, putative, expressed.- Oryza sativa (japonica cultivar-group).
	Q245R9_TETTH	Hypothetical protein.- Tetrahymena thermophila SB210.
	AAM72802	AE006470 NID: - Chlorobium tepidum TLS
	Q1NG56_9SPHN	Hypothetical protein.- Sphingomonas sp. SKA58.
	Q38AS5_9TRYP	Hypothetical protein.- Trypanosoma brucei.
	Q4CFT1_CLOTM	Hypothetical protein precursor.- Clostridium thermocellum ATCC 27405.
	Q4N8Y0_THEPA	Hypothetical protein.- Theileria parva.
	Q9ZTY0_EMIHU	Putative calcium binding protein.- Emiliania huxleyi.
	Q4LB11_9RODE	Erythropoietin receptor.- Spalax galili.
	Q4EB11_9RICK	Preprotein translocase, YajC subunit.- Wolbachia endosymbiont of Drosophila ananassae.
	Q26YD4_MYCFV	Transposase IS3/IS911.- Mycobacterium flavescens PYR-GCK.
	Q4DG98_TRYCR	Hypothetical protein.- Trypanosoma cruzi.
	Q69KB7_ORYSA	Hypothetical protein B1047H05.26 (Hypothetical protein OSJNBb0035K09.33).- Oryza sativa (japonica cultivar-group).

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 56 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Protein View

Match to: **E1007993** Score: **261**
SYNTHETIC PUC19 FABD1.3 SEQUENCE.- vectors.
Found in search of C:\Documents and Settings\jamesjdz\Desktop\RJ_260808.pkl

Nominal mass (M_r): **51653**; Calculated pI value: **8.80**
NCBI BLAST search of E1007993 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: vectors

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **15%**

Matched peptides shown in **Bold**

1 MKYLLPTAAA GLLLLAAQPA MAQVQLQESG PGLVAPSQSL SITCTVSGFS
51 LTGYGVNWVR QPPGKGLEWL GMIWGDGNTD YNSALKSRLS ISKDNSKSQV
101 FLK**MNSLHTD DTAR**YYCARE RDYR**LDYWGQ GTTVTVSSAS TKGPSVFPLA**
151 **PSSKSTSGGT AALGCLVK**DY FPEPVTVSWN SGALTSGVHT FPAVLQSSGL
201 YSLSSVVTVP SSSLGTQTYI CNVNHKPSNT KVDKKVEPKS SMKYLLPTAA
251 AGLLLLAAQP AMADIELTQS PASLSASVGE TVTITCRASG NIHNYLAWYQ
301 QKQGKSPQLL VYYTTTLADG VPSRFSGSGS GTQYSLKINS LQPEDFGSYY

351 CQHFWSTPRT FGGGTKLEIK R**TVAAPSVFI FPPSDEQLK**S GTASVVCLLN
401 NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE
451 KHKVYACEVT HQGLSSPVTK SFNRGES

Match to: **Q6PI81_HUMAN** Score: **199**
IGHM protein.- Homo sapiens (Human).
Found in search of C:\Documents and Settings\jamesjdz\Desktop\RJ_260808.pkl

Nominal mass (M_r): **53317**; Calculated pI value: **7.50**
NCBI BLAST search of Q6PI81_HUMAN against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens
Links to retrieve other entries containing this sequence from NCBI Entrez:
AAH41037 from Homo sapiens

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **7%**

Matched peptides shown in **Bold**

1 MELGLSWVFL VAILEGVQCE VQLVESGGGL VQPGGSLRLS CAASGFTFSS
51 YWMSWVRQAP GKGLEWVANI KQDGSEKYYV DSVKGRFTIS RDNAKNSLYL
101 QMNSLRAEDT AVYYCAREFE STMTTVNADY YYFYMDVWGK **GTTVTVSSAS**
151 **TKGPSVFPLA PSSKSTSGGT AALGCLVK**DY FPEPVTVSWN SGALTSGVHT
201 FPAVLQSSGL YSLSSVVTVP SSSLGTQTYI CNVNHKPSNT KVDKRVEPKS
251 CDKTHTCPPC PAPELLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE
301 DPEVKFNWYV DGVEVHNAKT KPREEQYNST YRVVSVLTVL HQDWLNGKEY
351 KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSREEMT KNQVSLTCLV
401 KGFYPSDIAV EWESNGQPEN NYKTTPPVLD SDGSFFLYSK LTVDKSRWQQ
451 GNVFSCSVMH EALHNHYTQK SLSLSPGK

Match to: **AAS85976** Score: **160**
AY393038 NID: - Homo sapiens
Found in search of C:\Documents and Settings\jamesjdz\Desktop\RJ_260808.pkl

Nominal mass (M_r): **18392**; Calculated pI value: **7.86**
NCBI BLAST search of AAS85976 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **15%**

Matched peptides shown in **Bold**

1 KPTQTLTMTC TFSGFSLSTS GVGVGWIRQP PGKALDWLAL IYWDDDKRYS
51 PSLKSRLTIT KDTSKNQVVL TMTNMDPVDT ATYYCAHRPD YYDSIGALFD
101 YWGQGTLVTV SSASTK**GPSV FPLAPSSKST SGGTXALGCL VK**DYFPEPVT
151 VSWNSGALTS GVHTFPAVL

Match to: **Q4CR60_TRYCR** Score: **72**
Trans-sialidase, putative (Fragment).- Trypanosoma cruzi.
Found in search of C:\Documents and Settings\jamesjdz\Desktop\RJ_260808.pkl

Nominal mass (M_r): **93181**; Calculated pI value: **5.03**
NCBI BLAST search of Q4CR60_TRYCR against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Trypanosoma cruzi

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **1%**

Matched peptides shown in **Bold**

1 MLSRVAAVKA PRTHNRRRVT GSSGRRREGR ESEPQRPNMS RRVFTSAMLL
51 LLLVVMMCCG SGGAAQAGVE EPSSESTFEW RGINDGGGET VESLGVPSLL
101 KVGSDVFAVA EAQCKNGGVS FTGVTSQPLK TQTAKTPVEV LKKPKDETQV
151 LEEGASEDQK KKVDVSRPTT AVKGSDIYML VGQYSSAAVG ASDAAQLGLL

201 LVKGSVNSGV ANNKKIDWKD TESSPQRLFE KQPDSWTRLI GSGGSGVKMK
251 DETLVFPVEG TKKKADGTEV DVKTVSLIIH SKDNTDWKLS KGMSDGGCSD
301 PSVVEWEDDK LIMMAACDGG RRRVYESGDK GESWTEALGT LSRVWGNNKK
351 GEEAKAVRSG FITATVGNDG DKRNVMLVTL PVYAEKNVEK GKLHLWLTDN
401 THIVDIGPVS GDDDDAAASS LLYNSGENTD ENNEEELIAL YEKKKDGGKP
451 SPGVVSVLLT EQLQRVKGVL TTWKEVDKRV SQLCTSLIAQ KERASTDDVC
501 GAVKITAGLV GFLSGKFSEN TWRDEYLGVN ATVTKERAEE VENGVKFTGS
551 GAGAEWPVGK QGENQLYHFA NYNFTLVATV SIDNVPEGNT PISLVGVKMN
601 GDENNVLLGL SYDSEKKWHV LCGDKTTTKL SSTLGAETPQ HVVILLKNGT
651 QGSVYVDGQR VGNEECALGN GESKEISHFY IGGDGANAEN KEGVSVTVTN
701 VLLYNRPLDD TEIAAFNPNK APIEVQVDGS IEGDAIQPSG GGRQEEPRQS
751 LGSSGAGVVP ASTVSSAK**TS SGGEGSATQL VK**EESSDGSK NVGGASSPGS
801 DAAVETGDRS TVQGDGSSET LVGTPATADA YAPDAEAMGH DGTAVNPGAS
851 ASSGADGETA EGTNGQEKKE IHAQNGGVKA A

Match to: **PH1422** Score: **71**
Ig heavy chain V region (clone P3-69) - human (fragment)
Found in search of C:\Documents and Settings\jamesjdz\Desktop\RJ_260808.pkl

Nominal mass (M_r): **15108**; Calculated pI value: **6.84**
NCBI BLAST search of PH1422 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **8%**

Matched peptides shown in **Bold**

1 EVQLVQSGAE VKKPGESLKI SCKGSGYSFI TYWIAWVRQM PGKGLEWMGI
51 IYPGDSDTRY SPSFQGQVTI SVDKSISTAY LQWSSLKASD TAMYYCARHG
101 MEYYYGSGSS DYYYYYMDVW GK**GTTVTVSS ASTQ**

Table 7.2 Mascot search results for the mass spectrometry analysis of the bottom band of lane 6 in figure 3.6.

User : Jonathan James
Email : j.d.james.1@bham.ac.uk
Search title :
MS data file : C:\Documents and Settings\jamesjdz\Desktop\New Folder\RJ_140808_2.pkl
Database : MSDB 20060831 (3239079 sequences; 1079594700 residues)
Timestamp : 15 Aug 2008 at 09:16:09 GMT

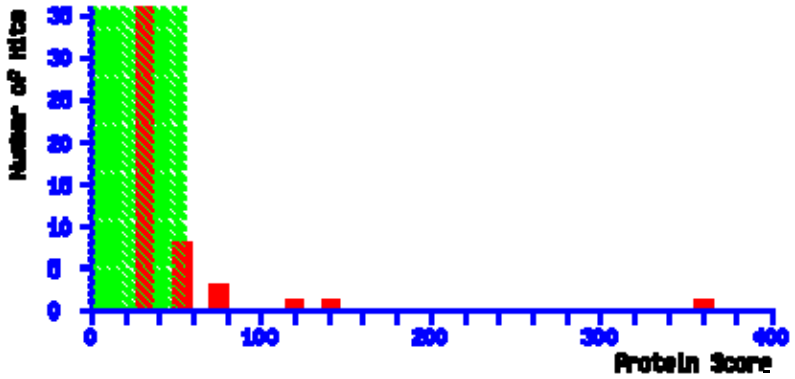
Protein hits :

E1007993	SYNTHETIC PUC19 FABD1.3 SEQUENCE.- vectors.
TRPGTR	trypsin (EC 3.4.21.4) precursor - pig (tentative sequence)
1BBJL	Fab' fragment of monoclonal antibody b72.3 (murineHUMAN CHIMERA), chain L - synthetic
AAA92435	REARRANGED IMMUNOGLOBULIN LIGHT CHAIN VARIABLE REGION (FRAGMENT).- Mus musculus (Mouse).
Q4CR60_TRYCR	Trans-sialidase, putative (Fragment).- Trypanosoma cruzi.
AAF05528	Immunoglobulin kappa light chain constant region (fragment).- Aotus nancymaae (Ma's night monkey).
TRRT1	trypsin (EC 3.4.21.4) I precursor - rat
BAC01690	Immunoglobulin kappa light chain VLJ region (Fragment).- Homo sapiens (Human).
K2C1_HUMAN	Keratin, type II cytoskeletal 1 (Cytokeratin-1) (CK-1) (Keratin-1) (K1) (67 kDa cytokeratin) (Hair alpha protein).- Homo sapiens (Human).
S24539	Ig kappa chain V region - mouse
AAA79897	MOUSE IG REARRANGED KAPPA-CHAIN MRNA FROM HYBRIDOMA (CBA/N X BALB.B), CLONE 7D6 (FRAGMENT).- Mus musculus (Mouse).
Q9Z1R9_MOUSE	Trypsinogen 16 (Protease, serine, 1).- Mus musculus (Mouse).
AAO84653	AF491643 NID: - Tachyglossus aculeatus
Q7S1V9_NEUCR	Predicted protein.- Neurospora crassa.
AAB50667	Idiotopes and metatopes of anti-fluorescein antibody 4-4-20-specific antibody (Fragment).- Cricetulus migratorius (Armenian hamster).
Q569I9_HUMAN	IGKC protein.- Homo sapiens (Human).
T07620	dnaK-type molecular chaperone hsp70 - Cyanophora paradoxa (fragment)
Q6NRB8_XENLA	LOC431933 protein (Fragment).- Xenopus laevis (African clawed frog).
Q2JE60_FRASC	Hypothetical protein.- Frankia sp. (strain CcI3).
Q1AUE5_9A	Lycopene beta and epsilon cyclase.- Rubrobacter xylanophilus DSM 9941.

CTN

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 55 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Protein View

Match to: **E1007993** Score: **360**
SYNTHETIC PUC19 FABD1.3 SEQUENCE.- vectors.
Found in search of C:\Documents and Settings\jamesjdz\Desktop\New Folder\RJ_140808_2.pkl

Nominal mass (M_r): **51653**; Calculated pI value: **8.80**
NCBI BLAST search of E1007993 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: vectors

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **22%**

Matched peptides shown in **Bold**

1 MKYLLPTAAA GLLLLAAQPA MAQVQLQESG PGLVAPSQSL SITCTVSGFS
51 LTGYGVNWVR QPPGKGLEWL GMIWGDGNTD YNSALKSRLS ISKDNSKSQV
101 FLKMNSLHTD DTARYYCARE RDYRLDYWGQ GTTVTVSSAS TK**GPSVFPLA**
151 **PSSKSTSGGT AALGCLVK**DY FPEPVTVSWN SGALTSGVHT FPAVLQSSGL
201 YSLSSVVTVP SSSLGTQTYI CNVNHKPSNT KVDKKVEPKS SMKYLLPTAA
251 AGLLLLAAQP AMADIELTQS PASLSASVGE TVTITCR**ASG NIHNYLAWYQ**
301 **QK**QGK**SPQLL VYYTTTLADG VPSRFSGSGS GTQYSLK**INS LQPEDFGSYY
351 CQHFWSTPRT FGGGTKLEIK **RTVAAPSVFI FPPSDEQLKS GTASVVCLLN**
401 **NFYPR**EAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE
451 KHKVYACEVT HQGLSSPVTK SFNRGES

Match to: **1BBJL** Score: **118**
Fab' fragment of monoclonal antibody b72.3 (murineHUMAN CHIMERA), chain L - synthetic
Found in search of C:\Documents and Settings\jamesjdz\Desktop\New Folder\RJ_140808_2.pkl

Nominal mass (M_r): **23345**; Calculated pI value: **6.34**
NCBI BLAST search of 1BBJL against nr
Unformatted sequence string for pasting into other applications

Taxonomy: synthetic construct

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **22%**

Matched peptides shown in **Bold**

1 DIQMTQSPAS LSVSVGETVT ITCRASENIY SNLAWYQQKQ GKSPQLLVYA
51 ATNLADGVPS R**FSGSGSGTQ YSLK**INSLQS EDFGSYYCQH FWGTPYTFGG
101 GTRLEIKR**AD AAPTVFIFPP SDEQLKSGTA SVVCLLNNFY PR**EAKVQWKV
151 DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG
201 LSSPVTKSFN R

Match to: **AAA92435** Score: **70**
REARRANGED IMMUNOGLOBULIN LIGHT CHAIN VARIABLE REGION (FRAGMENT).- Mus musculus (Mouse).
Found in search of C:\Documents and Settings\jamesjdz\Desktop\New Folder\RJ_140808_2.pkl

Nominal mass (M_r): **9272**; Calculated pI value: **10.14**
NCBI BLAST search of AAA92435 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Mus musculus

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **32%**

Matched peptides shown in **Bold**

1 TVTITCR**ASG NIHNYLAWYQ QK**QGKSPQLL VYNAKTLADG VPSR**FSGSGS**
51 GHNILSRSTA CSLKILGVIT VNIFGVLRGR SVEAPS

Match to: **AAF05528** Score: **66**
Immunoglobulin kappa light chain constant region (fragment).- Aotus nancymaae (Ma's night monkey).
Found in search of C:\Documents and Settings\jamesjdz\Desktop\New Folder\RJ_140808_2.pkl

Nominal mass (M_r): **9488**; Calculated pI value: **4.65**
NCBI BLAST search of AAF05528 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Aotus nancymaae

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **18%**

Matched peptides shown in **Bold**

1 AVAAPSVFIF QPSEEQVK**SG TASVVCLLND FYPR**DVSVKW KVDDVVQSSN
51 VQDSITEQDS KDNTYSLSST LTLSSTEYQR HKVYA

Match to: **BAC01690** Score: **51**
Immunoglobulin kappa light chain VLJ region (Fragment).- Homo sapiens (Human).
Found in search of C:\Documents and Settings\jamesjdz\Desktop\New Folder\RJ_140808_2.pkl

Nominal mass (M_r): **29191**; Calculated pI value: **7.60**
NCBI BLAST search of BAC01690 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **12%**

Matched peptides shown in **Bold**

1 MKYLLPTAAA GLLLLAAQPA MAEIVLTQLP GTLSLSPGER ATLSCRASQS
51 ISSSHLAWYQ QKPGQAPRLV IYGASNRATG IPDRFSGSGS GTDFTLTISR
101 LEPEDFAVYH CQQYDRSVVT FGSGTRLDIK R**TVAAPSVFI FPPSNEQLKS**
151 GTASVVCLLN NFYPREAKVQ WKVDNAPQSG NSQESVTEQD SKDSTYSLSS
201 TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGECSAR QSTPFVCEYQ
251 GQSSDLPQPP VNAGGGSGGG

Match to: **S24539** Score: **48**
Ig kappa chain V region - mouse
Found in search of C:\Documents and Settings\jamesjdz\Desktop\New Folder\RJ_140808_2.pkl

Nominal mass (M_r): **13087**; Calculated pI value: **8.64**
NCBI BLAST search of S24539 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Mus musculus

Fixed modifications: Carbamidomethyl (C)

Variable modifications: Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **11%**

Matched peptides shown in **Bold**

1 MKLPVRLLVL MFWIPASSSD VVMTQTPXSL PVSLGDQAXI SCRSSQSIVH
51 SNGNTYLEWY LQKXGQSXKL LIYKVSNRXS GVPDR**FSGSG SGTDXTLK**IS
101 RVEAEDXGVY YCFQGSHV

Match to: **AAA79897** Score: **47**
MOUSE IG REARRANGED KAPPA-CHAIN MRNA FROM HYBRIDOMA (CBA/N X BALB.B), CLONE 7D6 (FRAGMENT).- Mus musculus (Mouse).
Found in search of C:\Documents and Settings\jamesjdz\Desktop\New Folder\RJ_140808_2.pkl

Nominal mass (M_r): **12455**; Calculated pI value: **8.66**
NCBI BLAST search of AAA79897 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Mus musculus

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **11%**

Matched peptides shown in **Bold**

1 DVVMTQTPLS LPVSLGDQAS ISCRSSQSLV HSNGNTYLHW YLQKPGQSPK
51 LLIYKVSNRF YGAPDR**FSGS GSGTDFTXK**I SRGEXEDXGV YFCSQSTHVP
101 LTFGAGTXLE LKR

Table 7.3 Mascot search results for the mass spectrometry analysis of the bottom band of lane 1 in figure 3.6.

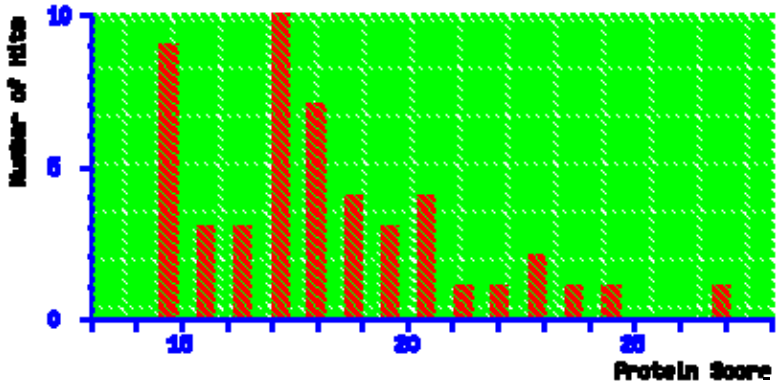
User : Jonathan James
Email : j.d.james.1@bham.ac.uk
Search title :
MS data file : C:\Documents and Settings\jamesjdz\Desktop\New Folder\RJ_140808_1.pkl
Database : MSDB 20060831 (3239079 sequences; 1079594700 residues)
Timestamp : 15 Aug 2008 at 09:13:13 GMT

Protein hits :		
	Q9DUB9_9VIRU	ORF4.- Torque teno virus.
	Q9XIL5_ARATH	Expressed protein (At2g15820).- Arabidopsis thaliana (Mouse-ear cress).
	Q73N03_TREDE	Rod shape-determining protein RodA.- Treponema denticola.
	Q5L186_GEOKA	TRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase.- Geobacillus kaustophilus.
	Q6CDE9_YARLI	Similarity.- Yarrowia lipolytica (Candida lipolytica).
	Q60WB1_CAEBR	Hypothetical protein CBG19196.- Caenorhabditis briggsae.
	Q8DIZ1_SYNEL	Malic enzyme.- Synechococcus elongatus (Thermosynechococcus elongatus).
	CAE22265	BX572101 NID: - Prochlorococcus marinus str. MIT 9313
	Q4FX43_LEIMA	Hypothetical protein.- Leishmania major strain Friedlin.
	Q4PBH3_USTMA	Predicted protein.- Ustilago maydis (Smut fungus).
	T35762	probable amino acid decarboxylase - Streptomyces coelicolor

Q4DFB5_TRYCR	Spliced leader RNA PSE-promoter transcription factor, putative.- Trypanosoma cruzi.
Q2J5D2_FRASC	Putative replication initiation protein.- Frankia sp. (strain CcI3).
Q36S26_MARHY	Flagellin.- Marinobacter aquaeolei VT8.
Q5FLG6_LACAC	Uridine kinase (EC 2.7.1.48).- Lactobacillus acidophilus.
Q3J1B1_RHOS4	Outer membrane efflux protein.- Rhodobacter sphaeroides (strain ATCC 17023 / 2.4.1 / NCIB 8253 / DSM 158).
Q28T66_JANSC	NADH-quinone oxidoreductase, F subunit (EC 1.6.99.5).- Jannaschia sp. (strain CCS1).
G6BPT4	baseplate protein gp6 - phage T4
Q6XA63_9BETA	IE1 protein.- Human herpesvirus 6.
Q2K920_RHIEC	Poly-beta-hydroxybutyrate polymerase protein.- Rhizobium etli (strain CFN 42 / ATCC 51251).

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 56 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



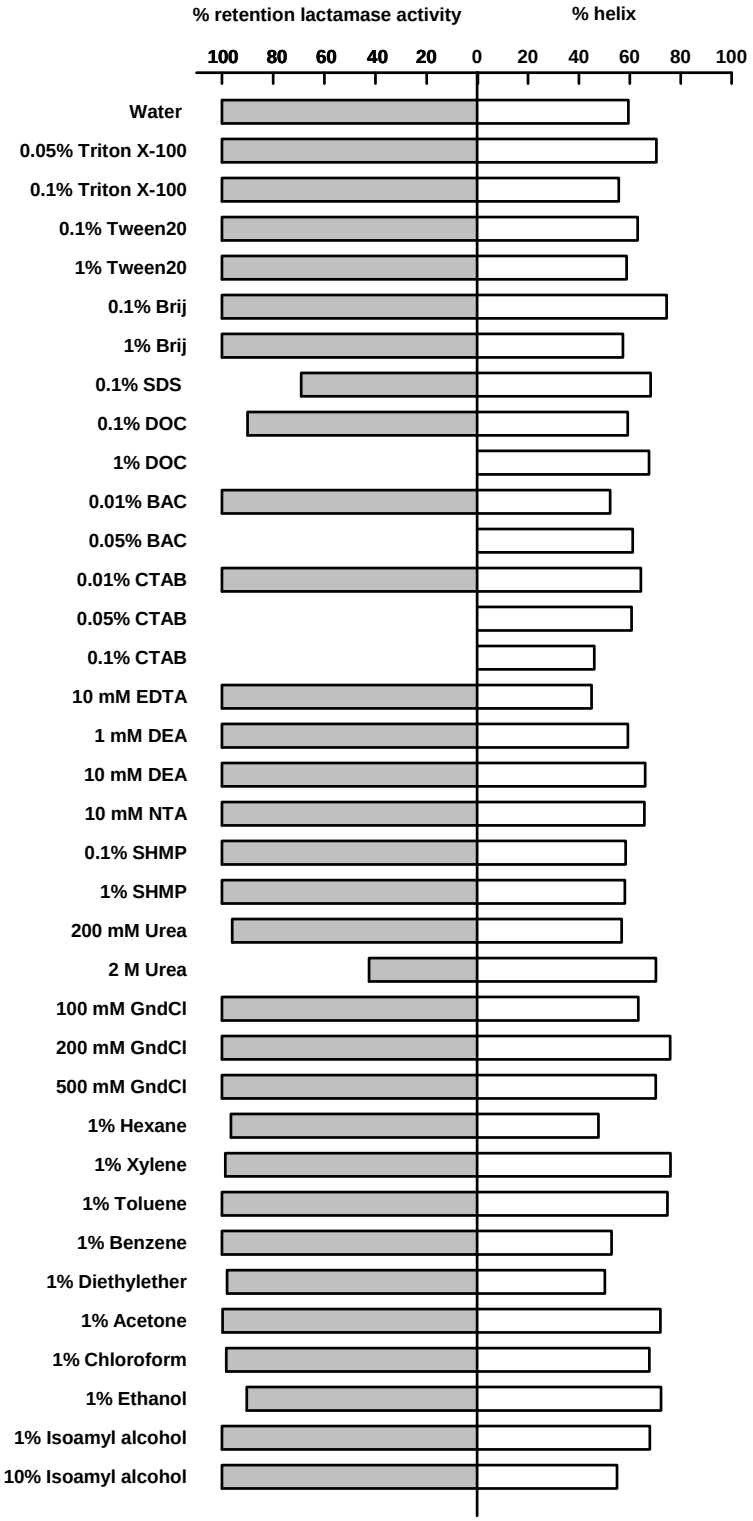


Fig. 7.5 Correlation of beta-lactamase activity and alpha-helix content in the presence of various chemicals.

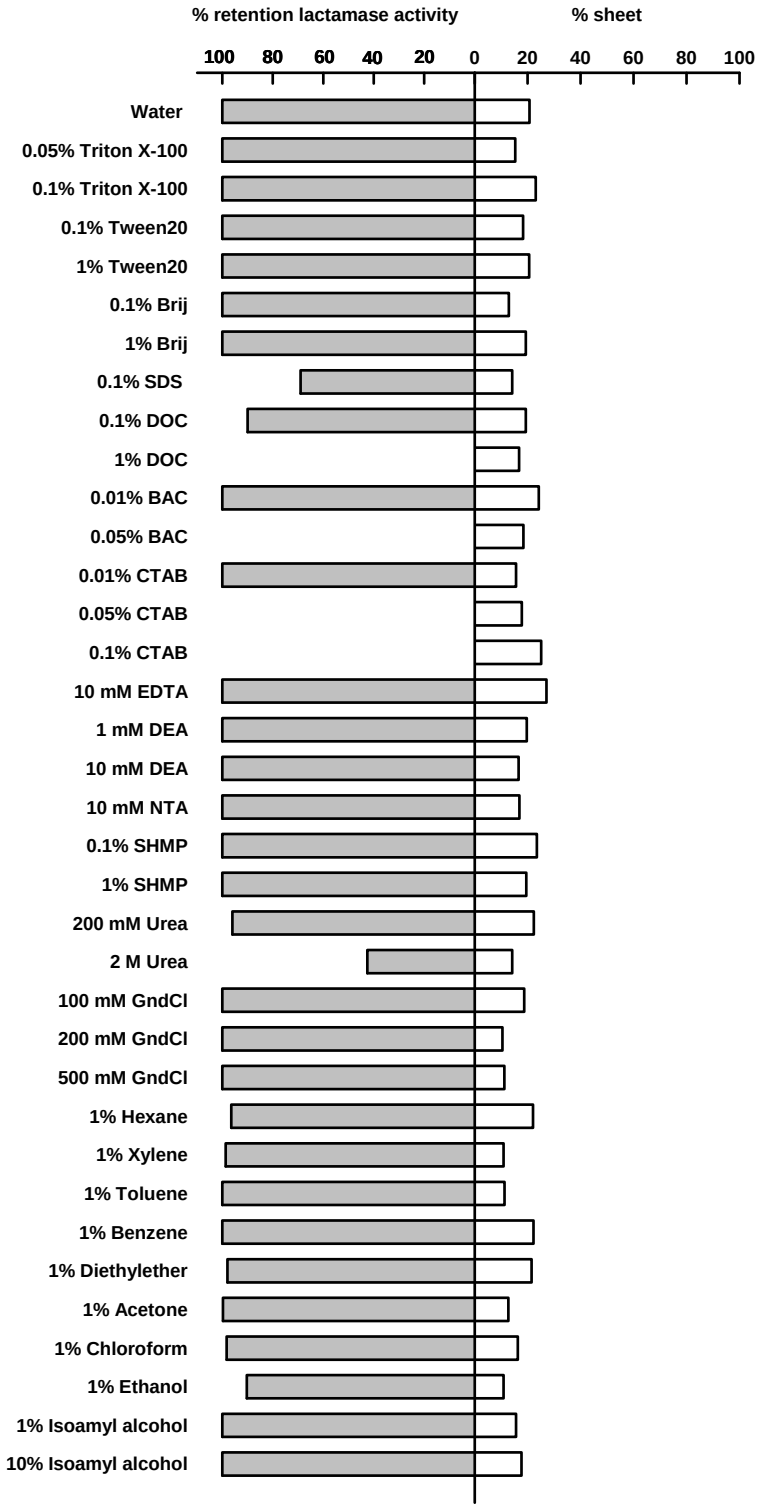


Fig. 7.6 Correlation of beta-lactamase activity and beta-sheet content in the presence of various chemicals.

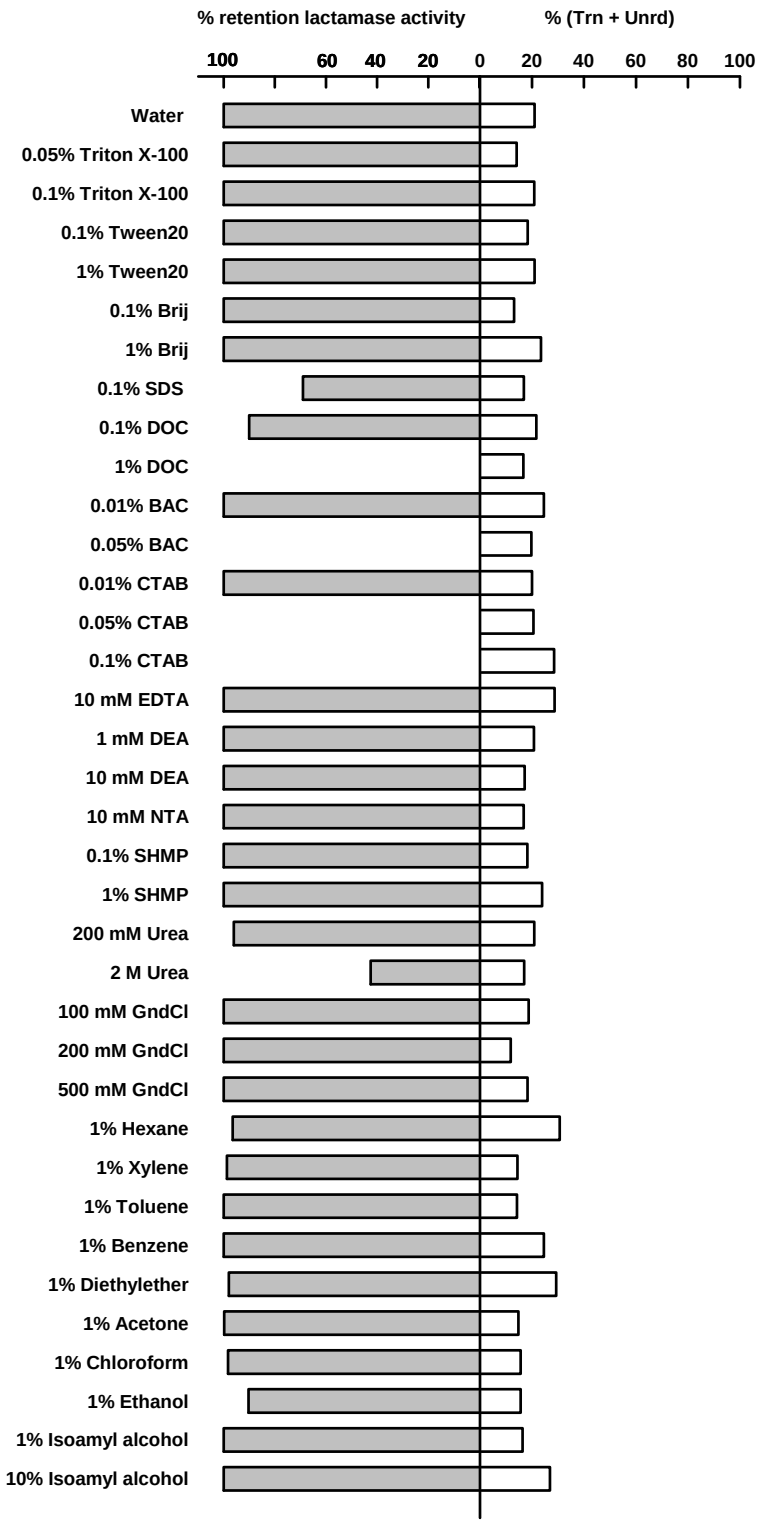


Fig. 7.7 Correlation of beta-lactamase activity and unordered content in the presence of various chemicals.

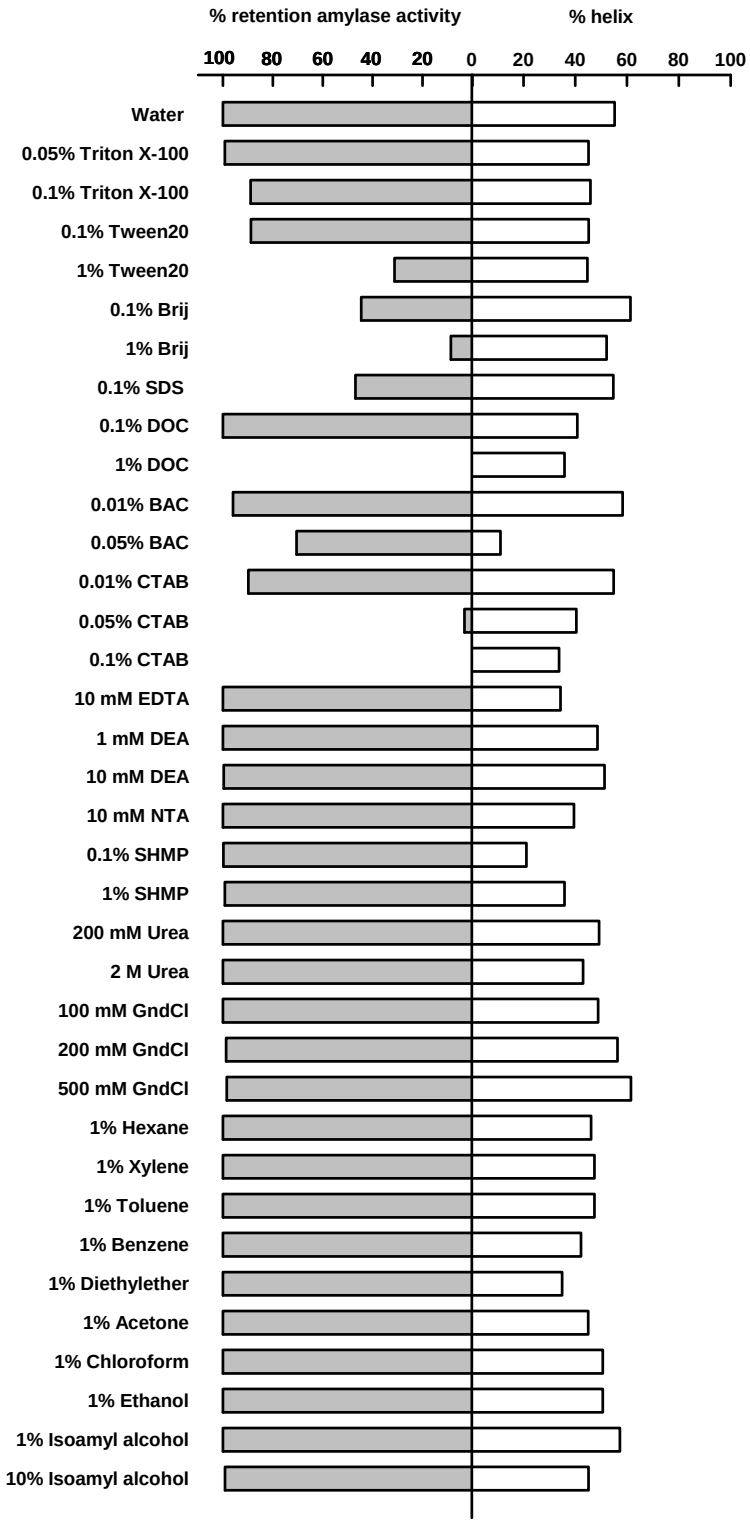


Fig. 7.8 Correlation of alpha-amylase activity and alpha-helix content in the presence of various chemicals.

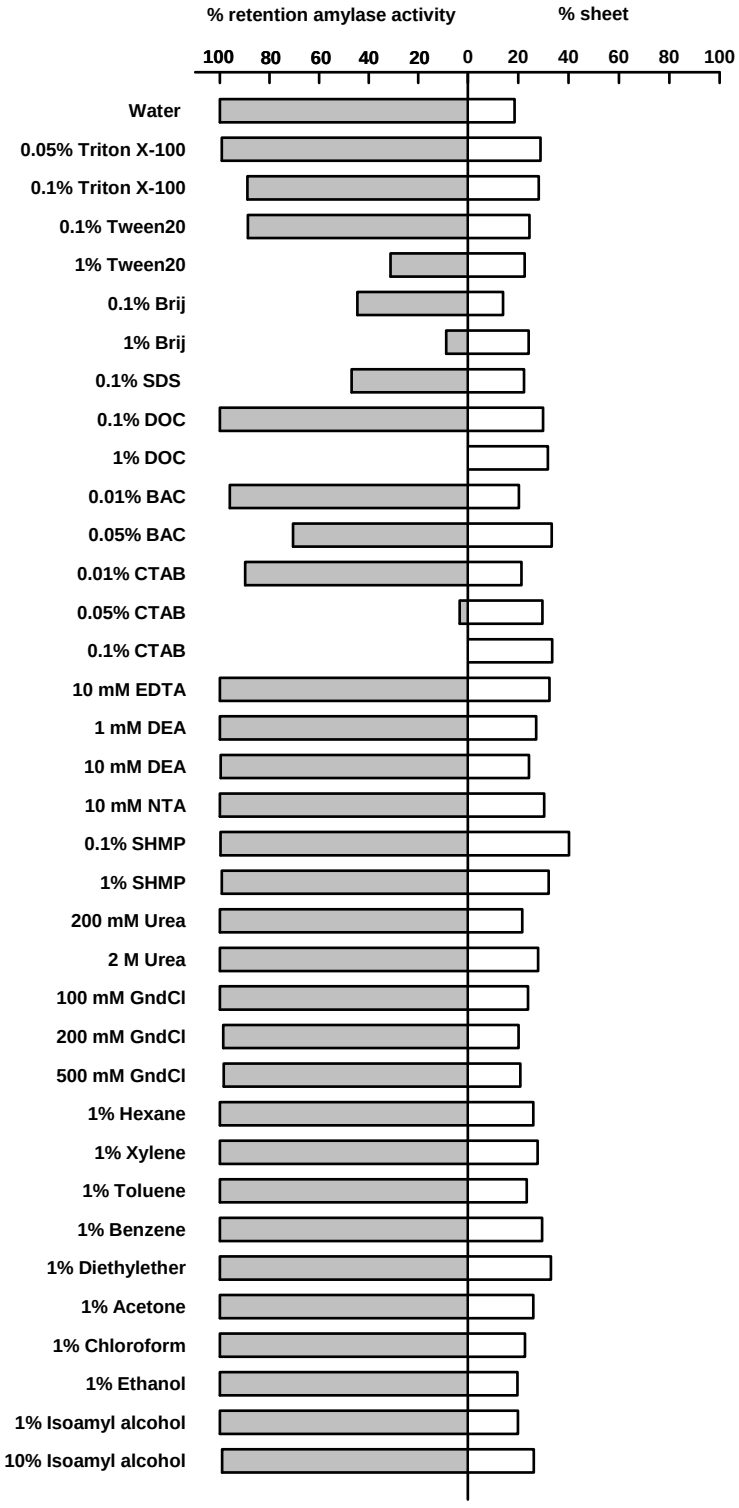


Fig. 7.9 Correlation of alpha-amylase activity and beta-sheet content in the presence of various chemicals.

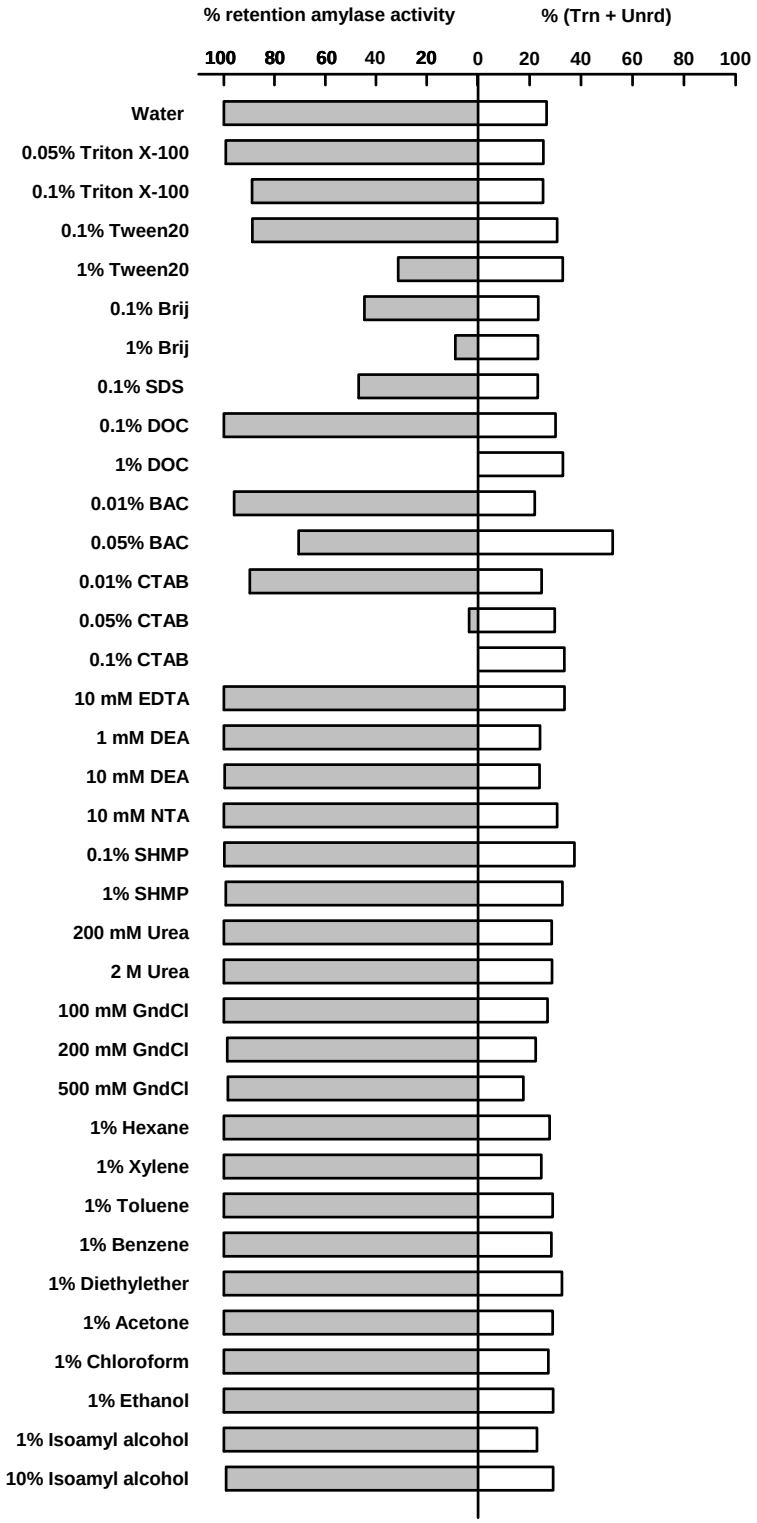


Fig. 7.10 Correlation of alpha-amylase activity and unordered content in the presence of various chemicals.

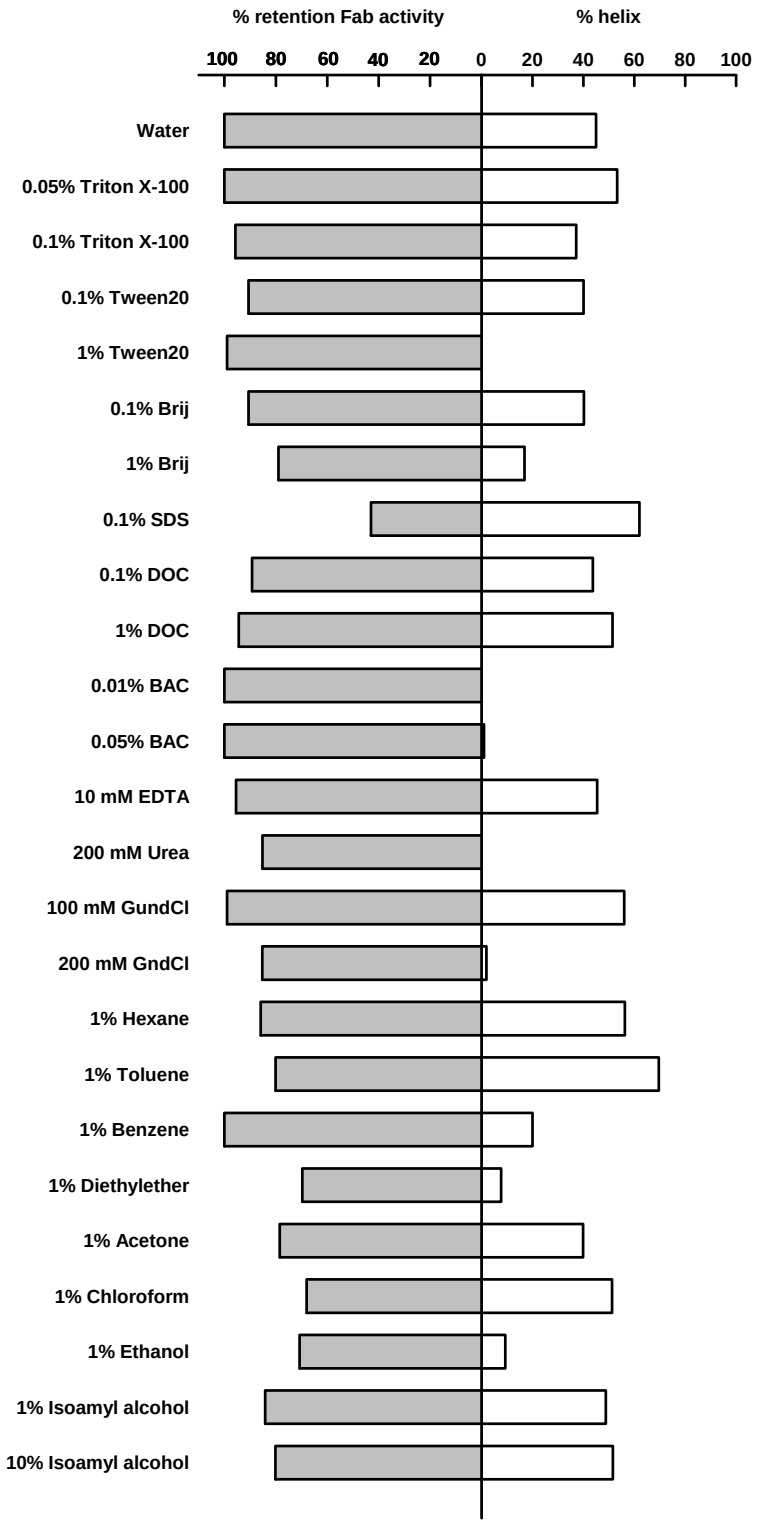


Fig. 7.11 Correlation of Fab D1.3 activity and alpha-helix content in the presence of various chemicals.

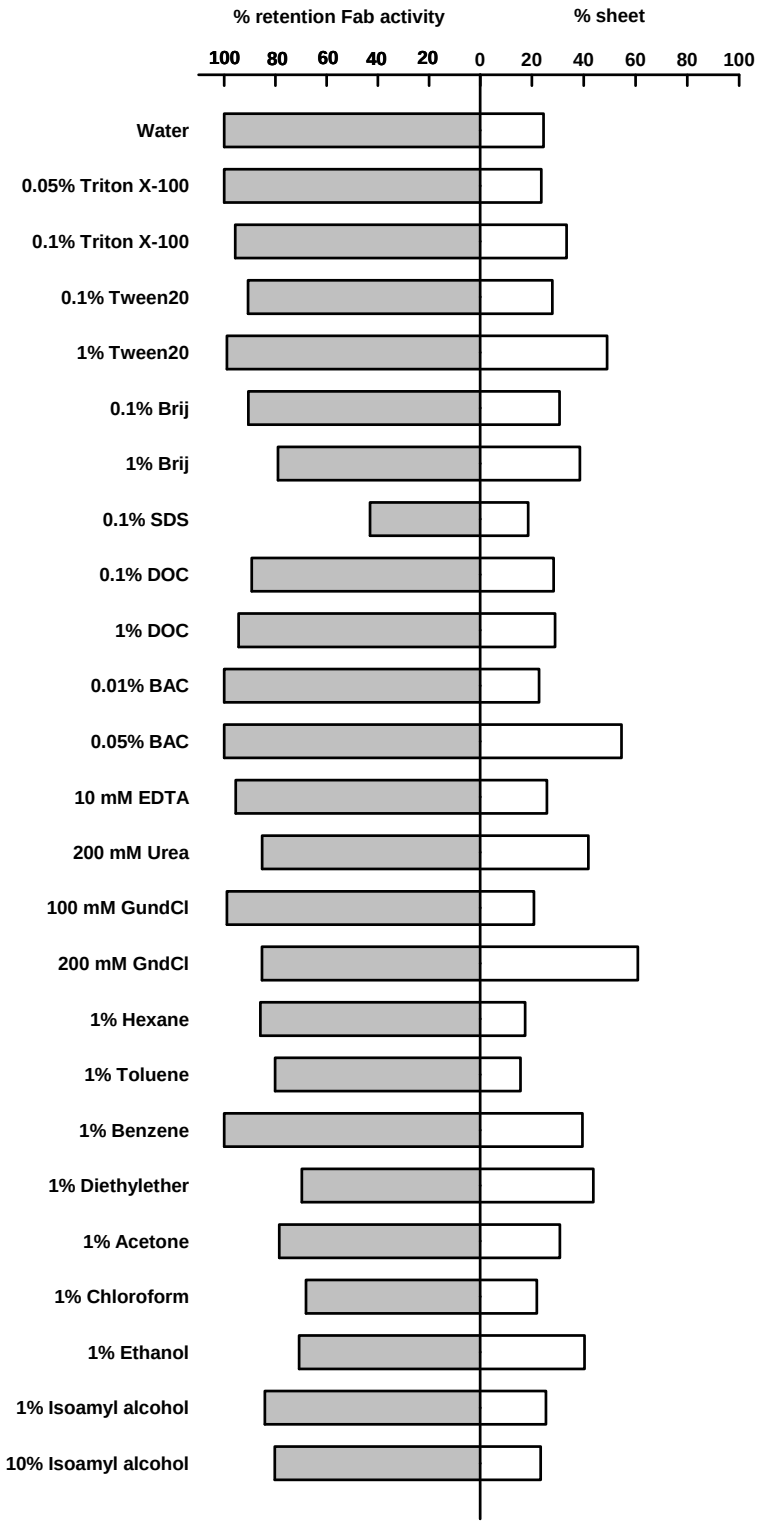


Fig. 7.12 Correlation of Fab D1.3 activity and beta-sheet content in the presence of various chemicals.

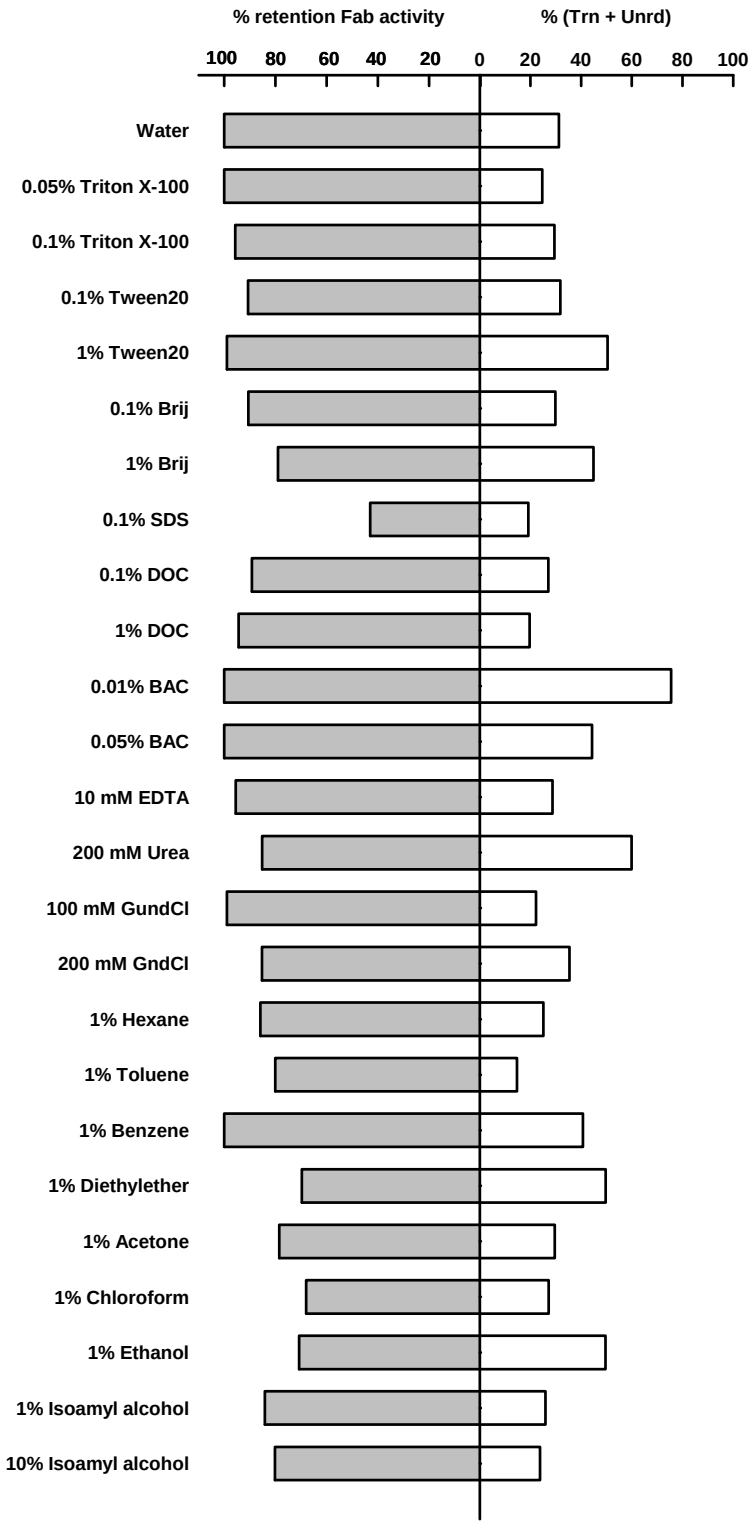


Fig. 7.13 Correlation of Fab D1.3 activity and unordered content in the presence of various chemicals.