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IMPROVEMENT OF DOWNSTREAM PROCESSING OF RECOMBINANT PROTEINS BY MEANS OF GENETIC ENGINEERING METHODS

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ABSTRACT

The rapid advancement of genetic engineering has allowed to produce an impressive number of proteins on a scale which would not have been achieved by classical biotechnology. At the beginning of this development research was focussed on elucidating the mechanisms of protein overexpression. The appearance of inclusion bodies may illustrate the success. In the meantime, genetic engineering is not only expected to achieve overexpression, but to improve the whole process of protein production. For downstream processing of recombinant proteins, the synthesis of fusion proteins is of primary importance. Fusion with certain proteins or peptides may protect the target protein from proteolytic degradation and may alter its solubility. Intracellular proteins may be translocated by means of fusions with signal peptides. Affinity tags as fusion complements may render protein separation and purification highly selective. These methods as well as similar ones for improving the downstream processing of proteins will be discussed on the basis of recent literature.

KEY WORDS

Downstream processing, recombinant protein, genetic engineering, fusion protein, fusion complement, purification tag, affinity handle, protein secretion, protein export.

INTRODUCTION

Proteins are the primary products of the translation of the genetic code. They are therefore of central interest for life science research as well as applications in a multitude of areas. Thus, they are a very important class of biotechnology-derived products. Enzymes have found a large area of applications due to their catalytic properties. Spectacular advancements in molecular genetics (regulation of transcription and translation, gene transfer, polymerase chain reaction *etc.*) and in the cultivation of animal and human cells (cell fusion, hybridoma-technique, transfection) have given the opportunity that intensive efforts are undertaken in order to develop processes for the production of species-specific peptide hormones (insulin, somatostatin, growth hormones, *etc.*), immunologically active proteins (interferons, interleukins, *etc.*), coagulation factors (urokinase, t-PA, factor VIII, *etc.*), as well as of vaccines and monoclonal antibodies.

Such proteins may only be obtained economically by means of biotechnological processes, since they represent polymers composed out of the 21 different proteinogeneous L-α-amino acids (including selenocysteine). Chemical synthesis of proteins [1] is only competitive for the production of short chain peptides. However, chemical synthesis is important when non-proteinogeneous amino acids have to be incorporated into peptides. The chemical route suffers from a laborious and expensive protection group chemistry and an error rate which is still too high. In addition, it has to be taken into account that proteins are only active in their natural folding state and that proteins of eukaryotes often need posttranslational modifications (glycosylation, proteolysis). In consequence, protein synthesis will need the use of microorganisms and of cells of animal or human origin. This also means that proteins will need to be separated from complex natural media. Cell-free translation systems are another possible method to produce recombinant proteins. The usefullness of such systems in bioprocessing is under investigation [274]. The importance of downstream processing very likely will at least remain or even increase [2, 3] because the separation and purification of proteins will remain the main source of costs for the manufacturing of proteins.

A whole spectrum of downstream processes has been developed for the recovery of proteins [4-7] and these have been documented in recent books and monographs [8-15]. Uncertainty with the use of novel processes, the partiality for problem-specific solutions and new possibilities for the production of a multitude of pharmacologically active proteins have considerably promoted the interest in downstream processing. Since the tool box of genetic engineering is more and more used for improving the production of proteins, it has been straightforward not only to use these techniques in order to achieve overexpression but to aim at the improvement of downstream processing as well. Quite some novel approaches for improving the downstream processing of proteins have already been worked out [16-19]. These novel methods may lead to the opportunity that very different proteins might be

purified by means of one unique process strategy. On the one hand, the physico-chemical properties of the proteins may be changed or amplified by means of protein design or site-directed mutagenesis. Recombination of different genes for the production of fusion proteins, on the other hand, is of primary importance because unique fusion partners may be chosen for the complementation of the target protein. Such fusion partners are adequate which are carriers of a specific affinity useful for the downstream process applied. The class of fusion proteins englobe the use of signal sequences in order to change the localization of the target proteins.

Such approaches and similar ones will be reviewed - taken from recent literature. The following chapter will deal with questions referring to the choice of host organisms and the requirement for producing biologically active natural proteins. Only a few issues can be emphasized with respect to host organisms and expression in general for not overloading the article. A further chapter discusses possibilities of manipulating the localization of proteins and the properties of cells which have direct impact on downstream processing. The last chapter is dedicated to strategies of protein modification for the improvement of protein separation and purification.

PRODUCT CONCENTRATION AND BIOLOGICAL ACTIVITY

The economy of downstream processing of proteins highly depends on the achievable product concentration. Therefore, recombinant protein production aims at the improvement of the overall protein concentration. However, aiming at a high product concentration may fail, unless a high proportion of biologically active proteins is obtained.

Overexpression

The first step for improving the concentration of the target protein is the choice of an adequate biological system by means of which overexpression may be achieved. Many such expression systems are available. For the optimisation of these systems a series of factors have to be taken into account referring to the replication, transcription and translation of the genes.

The replication of genes coding for recombinant proteins strongly influences the stable transmission as well as the copy number of these genes. Most expression systems for bacteria use plasmids as vehicles of recombinant DNA. Plasmid stability depends on both the structural and the segregative stability. The structural stability refers to the error rate during plasmid replication, whereas the segregative stability depends on the transmission of the plasmid on to daughter cells. Besides the correct replication of plasmids the structural stability is influenced by the rate of mutation and the gene repair facilities of the host organism. The segregative stability, commonly called plasmid stability, depends on a

series of factors which cannot be discussed here in detail [20]. Means for stabilizing or enhancing plasmid stability have been discussed by Kumar *et al.* [21].

The rate of gene expression may be accelerated by increasing the number of plasmids per cell. This may be achieved by using so-called multi-copy plasmids, the copy number of which may reach 100 owing to their particular mode of replication. Since the plasmid copy number has to be high only at the time when the target protein shall be produced, a controlled induction of plasmid replication represents an advantageous strategy. This may be achieved by using so-called runaway replication plasmids the copy number of which may easily reach 1,000 [22]. An obvious additional strategy consists of integrating several copies of the coding gene into the plasmid. However, it has to be kept in mind that a high plasmid copy number may affect the physiological state of the host organism leading to a limited rate of gene expression.

Both a promoter-operator system and an appropriate termination sequence are required in order to achieve efficient transcription. In addition, transcription should be inducible. During the development of expression systems different sequences have been recognized as strong promoters. A strong promoter is the prerequisite of a high transcription frequency. The best known inducible bacterial promoter-operator systems are those using operators derived from the *lac*- [23], *trp*- [24] and the λ- [25] systems. The *lac*-operator is often used for the induction of different promoters like *lac* and *tac*. Induction is achieved by the addition of IPTG (isopropyl-β-D-1-thiogalactopyranoside). Promoters coupled with the *trp*-operator may be induced by IAA (3-(3-indolyl)-acrylic acid). An induction by means of a temperature jump is applied in the case of λ-promoters using a temperature sensitive mutant strain with respect to the λ-repressor. Chemical inducers may be quite expensive and may lead to contamination of the product. Temperature induction may cause undesirable secondary products to appear due to an altered metabolism. Therefore, there is still a need for improved novel promoters [281] - like ones inducible by oxygen depletion [26] or by means of a pH-shift [279]. When strong promoters are applied, a strong terminator is required as well. Without an appropriate termination sequence transcription would proceed beyond the target gene and transcription would lead to useless products. Appropriate sequences like the *trpA*-transcription terminator are commercially available [e.g. 27].

Transcription is the first step during gene expression leading to mRNA. The second step involves the ribosomes which are responsable for the translation of the mRNA into the amino acid sequence of the recombinant protein. Translation efficiency depends on the stability of the mRNA, the ribosomal binding site (RBS), the correct termination of translation as well as the use of special codons. The stability of mRNA is a function of its susceptibility for hydrolysis by ribonucleases. It is commonly assumed that the lifetime of mRNA is influenced mainly by the presence of 3'-ribonucleases, because the secondary

structure of the 3'-terminal sequence of the mRNA is of crucial importance [28]. In consequence, mRNA stability may be enhanced by changing the 3'-terminal sequence or shifts in growth rate [264]. The lifetime of the mRNA determines how often it may be used by the ribosomes for translation [29]. Newest results suggest that RNA degradation may depend on 5'-terminal base pairing in *E. coli.* [221].

Recombinant genes are commonly introduced behind an inducible promoter, but they often have their own ribosomal binding site (RBS). This may be the reason for a low translation frequency, although the rate of transcription may be high. Optimized gene expression systems include an RBS especially designed for the particular host organism [30]. Thus, AT-rich sequences may be introduced up- and downstream of the RBS in order to increase the initiation frequency of translation [31]. The distance between RBS and the start codon AUG may also be changed in order to achieve higher productivities [32]. Synthetic RBS-sequences have not led to improvements of gene expression [33]. A strong terminator is required for efficient translation, too. Appropriate sequences are commercially available [27].

If a synthetic gene is used the sequence of which has been deduced from the amino acid sequence of a protein, the base sequences of the codons for the amino acids should be chosen with care. Studies with genes of highly expressed proteins have shown that certain codons out of the pool of the degenerate genetic code are preferred [34, 266].

Protein Stability

After translation the stability of the protein itself determines how much product will be obtained. Protein stability mainly depends on the susceptibility of the protein for proteolytic decomposition. Especially in the case of recombinant proteins, proteases may cause a considerable loss of product [35, 222]. It seems as if heterologous proteins were recognized as belonging to a different species with the consequence of a rapid proteolytic degradation. Different proteases may be responsible for protein digestion. In the case of *E. coli* the protease La coded on the *lon*-gene is accused of mainly being responsible for the degradation of recombinant proteins [36]. By using *lon*-minus mutants this problem may be partially overcome. However, the use of such mutants may cause a series of difficulties during cultivation. The product of the *lon*-gene is also controlling cell division and the development of the polysaccharide capsule. Therefore, lon-minus mutants tend to overproduce polysaccharides causing strong production of slime and interference with cell division and growth [35]. This problem may be circumvented by controlling the *lon*-gene by inducing the lon-function during the growth phase and switching it off during the production phase. However, this control is quite delicate since the concentration of the *lon*-gene product has to be kept in a very narrow band. This may not be achieved with commonly used promoters because of the constitutive gene expression taking place in spite of

repression [37]. By inactivation of structural and regulatory genes of the slime capsule synthesis it is possible to prevent slime production and the growth problems related with that [38]. Modifications of the *sulA*-gene the product of which regulates the lon-coded protease may yield a significant improvement of cell division for *lon*-negative strains. However, it has to be taken into account that *lon*-negative strains are very sensitive with respect to the induction of stress proteins causing additional growth problems.

Additional methods to reduce protein degradation are to inhibit the host-mediated degradation of foreign proteins by phage infection, using T4-promoter [262] and to consist the influence of growth rate on proteolysis [271]. An easier to implement strategy for reducing protein degradation consists in the synthesis of fusion proteins. Thus, small heterologous peptides which commonly are very labile have been stabilized by fusion with large proteins like β-galactosidase or *Staphylococcus* protein A [39-43, 272]. In the case of such fusions it may be necessary to split off the fusion complement in order to obtain the target protein in the desired form.

The export of proteins is another elegant possibility to avoid the intracellular environment rich of proteases [44]. However, some secretion systems may also export proteases into the extracellular environment. The problems related to the presence of proteases have to be considered especially for the operation of cell disintegration [45]. Therefore, the addition of protease inhibitors may be necessary during cell disruption.

Posttranslational Modification

Many proteins, in particular eukaryotic proteins of pharmaceutical interest, require further modification after protein biosynthesis. Such posttranslational modifications may consist in phosphorylation, glycosylation, the cleavage of sequences from pre-pro-proteins of especially secretory proteins as well as the cleavage of the primary protein chain. Without appropriate modification these proteins are often obtained in a biologically inactive form.

In many cases it is relatively easy to produce large quantities of protein by means of bacterial systems like *E. coli*. However, strains of *E. coli* are not provided with a pathway for the glycosylation of proteins. If glycosylation is of crucial importance, other less convenient expression systems have to be used. Yeast cells, simple eukaryotes, have glycosylating activity, but often they use other glycosidic residues then those required for the particular case [46]. There is certainly still a long way to go until bacterial systems or yeasts may have learned to achieve different posttranslational modifications by means of genetic engineering. Cells of animal and human origin will remain the systems of choice for proteins needing adequate posttranslational processing [4]. Another way to circumvent this problem is to look for the biologically active part of the target protein which, by circumstance, may not need further modification [47].

Inclusion Bodies, Renaturation and Protein Folding

Recombinant proteins are often found as insoluble aggregates in the cytoplasm [17, 48]. Extremely high protein concentrations due to overexpression may be responsible for this phenomenon [270]. These accumulations of solid insoluble proteins are called inclusion bodies [49]. The formation of these refractile particles may represent an advantage as well as a disadvantage for the production of recombinant proteins. Thus, inclusion bodies are relatively simple to be recovered from cell homogenates [17, 50, 51], and are protected from proteolytic cleavage. However, a great disadvantage must be seen in the fact that proteins bound in inclusion bodies are biologically inactive. Therefore, these highly insoluble protein aggregates have first to be denatured under extreme conditions in order to be solubilized [51, 52]. Subsequently, these solubilized proteins have to be renatured by means of adequate methods in order to be transformed into their biologically active form. This procedure may yield heavy losses which may be acceptable if a high overproduction goes in common with a high added value. Genetic engineering methods for the reduction of aggregate formation consist in forcing protein secretion as well as in changing specific properties of the target protein which may be responsable for the occurence of inclusion bodies. Wilkinson *et al.* [48] describe factors influencing the solubility of recombinant proteins. The critical factors englobe the protein concentration, the distribution of surface charge, the portion of particular secondary structures, the contents of cysteine and proline as well as the overall hydrophobicity. These properties may be changed deliberately by site-directed mutagenesis as well as by fusion of the target protein with an appropriate complement [53, 277]. Some approaches along these lines have been discussed by Kotewicz [54]. The most simple possibility for reducing or even preventing inclusion body formation seems to be by decreasing the rate of expression [52, 223], e.g. by lowering the temperature after induction [224].

In the case of heterologous proteins it is often inevitable that aggregates are formed and the whole procedure of solubilization and renaturation has to be applied [55]. The achievement of the native structure is the most crucial aspect for the production of recombinant proteins [46]. Achieving correct protein folding is a fundamental problem and of enormous economic impact [51]. Protein folding depends on a variety of different factors [56-58]. Certain additives like e.g. PEG [59] may improve folding [60]. However, the kinetics of folding reveals a rather complex pathway [225].

In recent years it has become clear that *in vivo* protein folding is assisted by catalytic protein complexes. Such proteins obviously play a crucial role for protein folding and assembly. They belong to the heterogeneous group of heat-shock proteins (hsp), and are now divided into two more or less distinct classes [226]. The class of the chaperonins belonging to the hsp10 and hsp60 families occur both in eubacteria, mitochondria and plastids, whereas the class of the hsp70 family seems to be present everywhere where

protein folding occurs. Evidence is also emerging for the presence of cytosolic chaperonins in archaebacteria as well as in eukaryotes [226, 228]. However, the term chaperone is generally used for proteins which assist in protein folding. Anyway, the division of chaperones into two classes seems to be a quite simplistic approach, because it has been shown in *in vivo* experiments that five chaperones cooperate in a sequential ATP-dependent folding pathway [229]. Both *in vitro* and *in vivo* studies of protein folding have already revealed quite some details about their action [61, 62, 230, 231, 267] and other proteins assisting in protein folding like peptidyl-prolyl cis-trans isomerase and protein disulfide isomerase [232, 234]. Such studies may finally lead to the fine-tuning of the cellular machinery of protein folding. This would certainly lead to a considerable improvement for the manufacturing of correctly folded recombinant proteins.

Cleavage of Fusion Proteins

Fusion proteins exhibiting the desired biological activity may be used as such, if there are no special regulations requiring them to be transformed into their natural structure, as it is still common practice for proteins of therapeutical use. In the latter case the cleavage of the fusion complement has to be accomplished. For this purpose, the amino acid sequence of the fusion protein has to be constructed with a specific cleavage side.

Fusion proteins may be cleaved by chemical or enzymatic means. A chemical cleavage may be relatively unexpensive, but the rather drastic reaction conditions may lead to non-specific cleavage and denaturation of the target protein. Enzymatic methods are generally preferred, because specific cleavage sites may be introduced between the target protein and the fusion complement [63, 235]. Over 200 protein-cleaving enzymes are listed in a book of B. Keil [269]. Costs for the cleavage procedure may be reduced considerably by the development of an adequate standard cleavage sequence for a specific proteolytic enzyme. Table 1 contains an overview of available chemical and enzymatic cleavage methods and their respective cleavage sequences.

Abbreviations used in Table 1:

BNPS-skatol = 3-bromo-3-methyl-2-(2-nitrophenyl mercapto)-3H-indol,
CAT = chloroamphenicol acyltransferase, HIV = human immunodeficiency virus,
ompA = outer membrane protein A, Kex 2 = yeast endoprotease

Table 1: Cleavage of fusion proteins

Chemical methods		
Cleavage agent	**Cleavage-site sequence**	**Ref.**
Hydroxylamine	Asn ⇓ Gly	[64]
Formic acid	Asp ⇓ Pro	[65]
Acetic acid	Asp ⇓ Pro	[66]
Cyanogen bromide	Met ⇓	[67]
BNPS-skatol	Trp ⇓	[68]
2-Iodo-benzoic acid	Trp ⇓	[69]
N-Chlorosuccinimide	Trp ⇓	[70]

Enzymatic methods		
Enzyme	**Cleavage-site sequence**	**Ref.**
Carboxypeptidase A	Poly His ⇓	[71]
Carboxypeptidase B	Poly Arg ⇓, Poly Lys ⇓	[72]
Chymotrypsin	Trp ⇓, Tyr ⇓, Phe ⇓	[73]
Collagenase	Pro-X ⇓ Gly-Pro	[74]
Dipeptidyl aminopeptidase	X-Tyr ⇓ (X not Pro)	[75]
Endoproteinase	Lys ⇓	[76]
Enterokinase	Asp-Asp-Asp-Lys ⇓	[77]
Factor X_a	Ile-Glu-Gly-Arg ⇓	[78]
HIV-1 protease	CAT- ⇓ -HIV-1 protease fusion, selfsplitting	[79, 80]
IgA-protease	Y-Pro ⇓ X-Pro; Y = Pro, Ala, Gly, Thr X = Thr, Ser, Ala	[275]
Kallikrein	Pro-Phe-Arg ⇓	[81]
Kex 2	-Lys ⇓ Arg	[278]
ompA-Signal-peptidase	ompA-signal sequence ⇓	[82]
Protein C	Phe-Thr-Phe-Arg, Leu-Ser-Thr-Arg, Pro-Glu-Leu-Arg	[268]
Renin	Tyr-Ile-His-Pro-Phe-His-Leu ⇓ Leu	[83]
S. aureus strain V8 protease	Glu ⇓	[236]
Subtilisin	Ala-Ala-His-Tyr ⇓	[84]
Thrombin	Arg-Gly-Pro-Arg ⇓	[85]
Trypsin	Arg ⇓, Lys ⇓	[86]
Ubiquitin peptidase	Ubiquitin ⇓ Relaxin α-chain	[87]

LOCALIZATION

The localization of proteins is obviously of major importance for downstream processing. If the protein is to be found in the cytoplasm, the concentrated cell mass has at first to be broken. The target protein, in consequence, has to be separated from a complex mixture of proteins, nucleic acids; eventually cellular compartments and debris. A more gentle cell disruption may be applied, if the target protein is secreted into the periplasm of Gram-negative bacteria. Obviously disruption is superfluous in the case of extracellular proteins.

Separation of Cells and Cell Disruption

Biomass is most often harvested by centrifugation or cross-flow filtration [6]. For *E. coli*, a genetic engineering approach has been developed for changing the properties of the cells in order to facilitate biomass recovery. A gene has been cloned which codes for a protein localized on the outer membrane surface and which is responsable for the flocculation properties. This modification resulted in a higher sedimentation velocity of the cells [88]. Similar interventions should also yield better properties for centrifugation and filtration.

Mechanical as well as non-mechanical techniques are applied for cell disruption on a large scale [6,89]. The mechanical methods comprise high pressure homogenization and bead milling. Both processes may exhibit considerable protein losses due to uncomplete protein liberation and thermal denaturation. Non-mechanical methods englobe chemical as well as biochemical processes. Cells may be permeabilized by organic solvents or by enzymatic lysis of the cell wall [90]. The use of organic solvents implies appropriate safety precautions [91], whereas enzymic processes are expensive on a large scale [90].

By means of genetic modifications it becomes possible to control cell lysis. The product of the *kil*-gene of the plasmid ColE1 may yield complete lyses of the cells [92]. A recent patent describes how the *kil*-gene, under the control of the *lac*-promoter may lyse the cells after induction with IPTG [93]. This strategy yields an expression system for which cell lyses may be induced under controlled conditions after the recombinant product has accumulated.

Based on the same principle, the lysis gene E of phage ΦX174 may be employed - under control of the λ-P_L-promoter [94]. In the presence of the temperature-sensitive λ-repressor cI897, cell lysis may be induced by increasing the temperature to 42 °C.

Secretion

Protein export may circumvent some problems inherent to the expression of recombinant proteins [95]. It has already been discussed that the formation of inclusion bodies and protein degradation due to cytoplasmic proteases may be prevented. In addition, some

proteins may be letal for a host when overproduced. Secretion obviously may reduce this phenomenon [82]. However, secretion also may yield uncorrectly folded proteins especially in the case of eukaryotic proteins synthesized by bacteria.

Nevertheless, protein secretion into the medium considerably simplifies downstream processing. In this case the cells may be separated from the medium by e.g. centrifugation and the proteins may be isolated from the supernatant. The early separation of unbroken cells leads to reduced contamination by other proteins or cellular constituents - an obvious advantage. A minor disadvantage of this strategy is the need for handling large volumes of liquids.

The transport of proteins into the periplasm of Gram-negative bacteria represents a special case of protein secretion. After separation of biomass the product may be liberated from the periplasm prior to being isolated out of a relatively small volume [265]. Contamination due to cytoplasmic constituents may be avoided by gently removing the cell capsule and the outer membrane. This strategy has actually gained much attention. The periplasmic space representing about 20 to 40% of the cellular volume in the case of *E. coli* is large enough for the accumulation of large amounts of proteins. Besides the advantage that proteins in the periplasm are protected against the attack by cytoplasmic proteases (but not against outermembrane bound proteases [244], the environment of the periplasm favours the correct folding of proteins.

The gene of the target protein has to be coupled with an appropriate signal leader sequence and the host organism has to be provided with a cellular transport system in order to allow secretion of proteins. In addition, the target protein should not show properties preventing secretion.

Signal Sequences: Signal sequences commonly are short N-terminal peptides which enable proteins to use the sorting and transport systems of a particular organism. In bacteria signal peptides with a length of 15 to 30 amino acids are found [96,97]. These structures represent positively charged leader sequences allowing proteins to cross membranes. It may be mentioned that hemolysin of *E. coli* carries its signal peptide at the C-terminus. Between signal peptide and core protein a cleavage site is found at which the signal peptide is cleaved off by means of a specific signal peptidase after transport of the core protein has occured.

Secreting proteins may show additional domains besides the signal sequence which may be necessary for a successful transport. In this category inner sequences are found which facilitate or even stop transport, like in the case of membrane proteins, giving rise to the development of an export competent conformation. Therefore, fusion with a signal peptide

Table 2: Signal leader sequences for protein secretion

Signal sequence	Target protein	M/P	Ref.
Host : ***Escherichia coli***			
amy	Amylase, *B. stearothermophilus*	M	[99]
bla	Proinsulin, human	-	[100]
	IgG of mouse	-	[101]
	β-Lactamase	M	[102]
	Epidermal growth factor, rat	M/P	[103]
	Triosephosphatase, chicken	M	[104]
cgt	alk. Phosphatase, α-amylase	M	[282]
lamB	CD4 receptor of HIV	OM	[261]
malE	Gene 5 protein, phage M13	P	[105]
	Klenow-polymerase	P	[106]
	Nuclease A, *S. aureus*	P	[106]
ompA	Colony stimulation factor, human	-	[107]
	Superoxide dismutase, human	P	[82]
	Interferon α2	-	[108]
	Antiviral protein, *Mirabilis*	M	[109]
	α-Sarcin	P	[110]
	Prokallikrein, human	P	[111]
	Nuclease A, *S. aureus*	P	[82]
ompF	β-Endorphin	M	[19]
pelB	Antibody VH-domain, mouse	OM	[276,280]
phoA	Trypsin inhibitor, bovine	-	[112]
	Epidermal growth factor, human	-	[113]
	Fusion : β-galactosidase-alk. phosphatase	M/P	[98]
	α-neo-Endorphin	P	[114]
	Fusion : MBP-β-galactosidase	P	[115]
	Ribonuklease T1	P	[116]
phoS	Growth hormone release factor, human	P	[117]
spA	Parathyroid hormone, human	M	[118]
	Insulin-like growth factor, human	M	[119]
Ovalbumin	Ovalbumin	-	[120]
Pullulanase	β-Lactamase	M	[121]

Table 2: Signal leader sequences for protein secretion (continued)

Host : *Escherichia coli* (cont.)			
Preproinsulin (rat)	Proinsulin, rat	-	[122]
Enterotoxin LTA	Epidermal growth factor, human	-	[123]
Synthetic	Interferon α2	P	[124]
Metalloprotease	Metalloprotease, with helper protein	M	[125]
BRP	Insulin-like growth factor, human	M	[19]
	β-lactamase & α-amylase	M	[283]
	-	M	[126]
hly	Hemolysin	M	[127]
	Pseudomonas cholesterol esterase	M	[246]
Host : *Bacillus subtilis*			
alk. Protease *B. amyloliquefaciens*	alkaline Phosphatase	M	[128]
amy, *B. amyloliquefaciens*	Amylase, *B. licheniformis*	M	[129]
amy, prepro-peptide	Amylase & human growth hormone	M	[247]
bla, *E. coli*	Amylase, *B. licheniformis*	M	[129]
Prepro-neutral-protease	Growth hormone, human	M	[130]
Host : *Saccharomyces cerevisiae*			
Yeast killer toxin	α-Amylase, mouse	M	[248]
Prepro-α-factor	Viral proteins, human papillomavirus	M	[131]

Abbreviations used in Table 2:
amy = amylase, bla = β-lactamase, BRP = bacteriocin release protein, cgt = cyclodextrin glycosyltransferase of *B. circulans*, hly = hemolysin, M = secretion into the medium, malE = maltose binding protein, MBP = maltose binding protein, OM = secrection into the outer membrane, ompA = outer membrane protein A, ompF = outer membrane protein F, P = secretion into the periplasm, phoA = alk. phosphatase, phoS = phosphate binding protein, spA = *Staphylococcus aureus* protein A

may not be sufficient for the secretion of the fusion protein [98]. Table 2 contains a list of some signal sequences together with the target proteins for which they have been used.

E. coli is of interest as a secreting host organism, though even proteins naturally occuring in *E. coli* are hardly secreted. Known signal sequences like *bla* (β-lactamase), *malE* (maltose binding protein), *ompA* (outer membrane protein A) and *phoA* (alkaline phosphatase) originate from proteins being transferred into the periplasm of *E. coli*. Fusion proteins carrying these sequences are accordingly exported into the periplasm. However, this may not be true in any case. Even secretion into the medium has been observed. Thus, the structure of the fusion protein determines to quite some extent if a successful secretion may be achieved. Recent studies show evidence for the presence of a signal recognition particle in *E. coli* in analogy to eukaryotes [237, 238]. The signal sequences *spA* (*Staphylococcus* protein A) and *malE* are of particular importance since both may be used at the same time as affinity tags for facilitating the separation of the fusion protein.

Secretion Systems: Different organisms are used for aiming at secretion of proteins. Systems of bacterial origin include e.g. *Bacillus subtilis* [239], *Staphylococcus aureus*, *Streptomyces lividans* and last but not least *E. coli* [96]. Important eukaryotic secretion systems comprise different species of yeast like *Saccharomyces cerevisiae* [240], *Hansenula polymorpha* [241], *Kluyveromyces lactis* [242], *Yarrowia lipolytica* [243], of fungi like *Aspergillus oryzae*, and cellular strains of animal and human origin.

Not only the signal sequences used and the structural properties of the target protein are important in order to achieve successful secretion, but also the transport system and the composition of the cell membrane and the cell wall of the host organism.

There is increasing evidence that protein folding and protein export are competing processes in procaryotic cells. Disulfide bonds formed in the cytoplasm can lead to secretion incompetence [273]. In the case of *E. coli* there exists the possibility to gain control over the expression of certain proteins involved in the pathway of protein secretion [239]. These proteins belonging to the family of the *sec*-gene products can be expressed in parallel with the recombinant proteins in order to achieve an improved transport [97]. Modifications at the level of the cell membranes of *E. coli* lead to the appearance of so called leaky-mutants. With such mutants excretion of periplasmic proteins like alkaline phosphatase [132], β-lactamase [133], but also eukaryotic proteins like pro-insulin from rat [134] into the medium has been achieved. Therefore, the choice of an appropriate microbial strain [244] or cell line [245] represents an important merible with respect to the export competence. Another possibility consists in amplifying the amount of porin found in the outer membrane of *E. coli* [135]. However, such mutations often show the disadvantage of disturbing cell growth. Another interesting variation of this principle is the cloning of the

bacteriocin release protein (BRP). Human growth hormone which accumulated in the periplasm of *E. coli* was able to be exported into the medium upon induction of BRP [19].

MODIFICATION OF PROTEINS FOR IMPROVEMENT OF SEPARATION AND PURIFICATION

The separation and purification of proteins may be improved either by altering or by amplifying certain physico-chemical properties of the target protein by means of site-directed mutagenesis or by introduction of an affinity tag by means of a fusion complement. Adding a peptide or a protein as an affinity tag to the N- or C-terminus seems to be particularly attractive, since the tertiary structure of the target protein has not to be known and the primary structure is kept unchanged. In the case of a site-directed exchange of amino acids it should be known that these mutations occur at the surface of the protein in order to have an effect on downstream processing. In addition, changing the primary structure may affect the biological activity of the product. In general, protein fusion is easier to be accomplished than site-directed mutagenesis.

Most examples from literature are therefore to be found under the topic of fusion proteins. The fusion complement should exhibit a strong affinity for the complementary ligand used in the main process of separation. The advantage of the fusion method lies in the fact that in principle one generally applicable downstream processing strategy may be developed for a multitude of different target proteins. The additional cost for genetic engineering may be easily cushioned by savings on process development. Fusion proteins synthesized for the improvement of downstream processing often consist, beside the target protein and the affinity carrying protein, still of a signal leader sequence which should cause secretion of the fusion product into the periplasm in the case of Gram-negative bacteria or into the medium. Additionally, a peptide representing a specific cleavage site may be introduced between the target protein and the affinity carrying protein, if the desired protein has to be obtained in its native state. The particular problems of secretion an cleavage of fusion proteins has already been discussed in previous chapters.

Such fusion proteins will in future certainly be more and more commercialized without prior cleavage, if the biological activity of the target protein is not affected and if the fusion complement has no adverse effect on the particular application. It should also be taken into consideration that fusion proteins, that means proteins with two or multiple functions, are of strong interest for innumerable applications in areas such as general analytics, diagnostics, pharmaceutics as well as in health care [e.g. 19, 136, 137]. These multifunctional proteins are, however, so special in composition - e.g. an antibody fragment linked with a reporter enzyme - that an additional fusion with an affinity carrying protein may be envisaged in order to facilitate its recovery and purification [249]. It should be added that the construction of a fusion protein consisting of a purification tag and a

multiple repeat of the target protein each time separated by a cleavage site has also been proposed [250].

Protein-Ligand Interaction

It is important for the success of downstream processing strategies that fusion complements exhibit a strong specific interaction with the appropriate ligands used in the main separation procedure. The aim should be to obtain the fusion protein with near absolute purity in a single separation step. Appropriate pairs of combination of fusion complements and ligands are gathered in Table 3. The fusion complements carrying the site of affinity interaction are commonly called affinity handle, purification tag or affinity tag.

Table 3: Potential combinations of complementary interactions for improving downstream processing of proteins

Modification of the target protein or fusion complement (peptide or protein)	Interacting ligands on the part of the separation process (not restricted to peptides and proteins)
Antigen	Antibody or antibody-fragment
Antibody or antibody fragment	Antigen
Enzyme or enzyme binding site	Substrate, inhibitor or substrate analogue
Substrat, inhibitor or substrate analogue	Enzyme or enzyme binding site
Receptor or receptor fragment	Hormone
Hormone	Receptor or receptor fragment
Lectin or lectin fragment	(Poly-)saccharide
Binding protein	Binding ligand (e.g. DNA, RNA ...)
(Poly-)histidine	Metal-chelate ligand
(Poly-)lysine, (poly-)arginine (poly-)glutamate, (poly-)aspartate	Charge ligands, Ion exchangers
(Poly-)phenylalanine	Hydrophobic ligand
(Poly-)cysteine	Thiol group

Purification tags obviously have to be peptides or proteins, whereas ligands may be derived from any source biological or chemical. The term affinity interaction is used for the capacity of complex formation between antibody and antigen (immunoaffinity), between receptor and messenger as well as between enzyme and substrate or inhibitor. The complexation of enzyme with substrate analogues and of e.g. (poly-)histidine sequences with chelating ligands is often called pseudo-affinity interaction. However, the term affinity is nowadays used in a quite broad sense.

Affinity-based separation methods are state of the art in downstream processing of proteins [138]. However, only affinity chromatography has been developed so far that a multitude of ligands bound on solid phases are commercially available [139]. Very high specificities may be obtained with monoclonal antibodies raised against fusion complements. Since the production of antibodies is quite expensive, the application is restrained to the recovery of expensive proteins which have to be obtained in extremely high purity. In this case fusion proteins offer the possibility that antibodies raised against the fusion complement may be used for the recovery of quite different target proteins. Thus, the costs for development and production of the antibody may be settled against the benefit from several products. A new trend is the expression of immunologically active antibody fragments in bacteria [140]. This should considerably diminish the costs of production of specific antibody activities. This would also allow for a reversal of the commonly used method leading to the situation that the fusion complement may be the antibody and the ligand may act as the antigen. However, a problem commonly accountered for using the principle of immuno-affinity is that the antibody-antigen complexes only dissociate under quite drastic conditions.

Enzymes and their binding sites are quite convenient fusion complements, if these are known to form stable complexes with natural or synthetic substrates or inhibitors, respectively. A reversal of this principle is possible if a peptide may be identified as a potent inhibitor. All kinds of substrate affinity are quite important for downstream processing of enzymes. The most extensive experiences for the use of affinity interactions have been gathered in this area. Reactive dyes have found particular attention as chemically stable ligands [141]. These had been introduced mainly as coenzyme analogues into affinity separation techniques. Quite a number of this ligands bound to solid phases are commercially available. In principle, there is obviously an innumerable number of candidates on both sides, the purification tag and the ligand.

Highly specific interaction may be expected from the combination of a receptor and its respective messenger. If the messenger is a peptide hormone the principle may be reversed.

Saccharides are potent ligands binding to appropriate lectins. If the gene of the appropriate lectin is known, this interaction may be used in protein recovery as well.

The class of binding proteins shall comprise all those proteins which do not fit into the categories already mentioned. Examples for such proteins are those which exhibit pseudo-immuno-affinity interactions like the *Staphylococcus* proteins A [142] and G, which bind to specific gene sequences (DNA or RNA) as well as proteins which interact with certain chemical groups or molecules. New methods from molecular genetics like reverse transcription and the polymerase chain reaction allow for producing (short chain) RNA, which binds certain proteins or peptides with high specificity [143,144], by repeated cycles of mutation/selection/amplification. This technique might be used in order to synthesize specific (RNA-) ligands for any peptide which is to be used as a fusion complement.

An extremely interesting method for improving the separation and purification of proteins is based on the interaction of metal chelate ligands with, particularly, histidine side chains of proteins, because it is rather simple to be applied. Although this interaction is known since 1975, it has only found deeper interest because of the evolving genetic engineering methods. These methods permit either to introduce histidine residues at the surface of proteins by site-directed mutagenesis or to introduce a histidine-rich sequence at the terminus of the target protein. With respect to the specificity of the interaction it is quite helpful to know that histidine represents one of the most rare proteinogeneous amino acids. The most commonly used chelating ligand is the three-dentate iminodiacetic acid (IDA). Appropriate supports are easily derivatized with this chelating group [145] and respective chromatographic phases are offered by several companies [146]. Additional chelating ligands are N,N,N'-Tris (carboxymethyl) ethylenediamine (TED) [145], nitrilotri-acetic acid (NTA) [147] - actually N-(5-amino-1-carboxypentyl) iminodiacetic acid, a lysine derivative - and ethylenediamine-N,N'-diacetic acid (EDDA) [148]. Metal ions which have most commonly been used comprise Cu(II), Ni(II), Zn(II) and Fe(III). Cu(II)-IDA groups form already stable complexes in the presence of only one accessible histidine side chain, whereas for Zn(II)-IDA two vicinal histidine residues are required [149]. However, recent results have shown that there are exceptions from this simple rule [150]. The Ni(II)-NTA group seems to be particularly suited to complexing proteins with several histidine residues in series [151]. The chelate-protein complexes are stable in the neutral pH-range. Complex dissociation may be forced by decreasing the pH or by means of adding ammonium ions, imidazole, histidine or other complexing agents which compete with the protein for the chelating group. The application of this principle in chromatography is known under the term immobilized metal affinity chromatography (IMAC) which is well documented [146, 149]. For modification of the target protein, peptides of several histidine residues are the obvious fusion complements to be used in order to achieve a high selectivity of separation.

Altering the properties of proteins by means of fusion with amino acid oligomers or with self-repeating amino acid sequences can be done quite easily by genetic engineering. Oligomers of amino acids with charged side chains (lysine, arginine as well as glutamic-

and aspartic acid) may be used in order to recover proteins selectively by means of oppositely charged groups like ion exchangers. Hydrophobic interaction may be used for the recovery of proteins derivatized with oligomers of hydrophobic amino acids. The reactive thiol group of cysteine is able to form reversible covalent bonds with ligands.

Downstream Processing

Separation processes and purification steps of particular interest for downstream processing of recombinant proteins shall be discussed in this chapter. In principle, this area is well documented [e.g. 8-15, 152], however, it evolves rapidly. With increasing amount of proteins of high purity, large scale processing [13, 153] and continuous operation [5] are gaining considerable interest.

A survey on processes applied for the separation and purification of recombinant proteins is given in Table 4. The largest choice of commercially available solid supported ligands can be found for processes based on adsorption. A rich chemistry for the activation of solid surfaces and polymer matrices is known in order to bind particular ligands in such a way that they may interact with proteins. Therefore, chromatography is the most commonly used technique for protein separation and purification. Unfortunately, the term chromatography is used nowadays for quite a number of different techniques.

Table 4 : Processes for downstream processing of proteins

1	Solid-phase extraction
1.1	Adsorption
1.2	Chromatography
2	Liquid-phase extraction
2.1	Aqueous two-phase extraction
2.2	Microemulsion two-phase extraction
3	Precipitation
4	Membrane processes
4.1	Cross-flow filtration (micro- or ultrafiltration)
5	Electrophoretic processes

If proteins are modified by genetic engineering with respect to downstream processing, it is in most cases done by introducing affinity interactions with the aim that a stable complex of the protein and a carrier-fixed ligand may be obtained . The respective adequate processing step is commonly called chromatography, also called replacement chromatography, but represents a process of adsorption/desorption. By means of this technique, the capacity of the solid phase can be used to a maximum. The separation selectivity is optimized by the choice of different eluents applied in series. Elution is mainly carried out in fixed beds in order to achieve a concentrating effect as high as possible in addition to separation owing to the small degree of backmixing, whereas charging may happen as well in well-mixed apparatus [154] or in fluidized beds [155]. Well-mixed systems are particularly suited, if the target protein is to be recovered in the presence of biomass or cell debris [156-158]. Since the diffusional resistance of proteins is quite high, small macroporous particles have to be applied. However, the operation of beds of small particles may be limited by a high pressure drop. Therefore, columns with small aspect ratio (e.g. radial-flow columns) have gained considerable attention. In last consequence this leads to the application of functionalized porous membranes [159-161].

In the area of liquid phase extraction, aqueous two-phase systems have proved successful not only for the separation of biomass and cell debris but also for the separation of proteins [e.g.15, 162]. Since phase separation occurs due to the incompatibility among water-soluble polymers or of these with salts, affinity interactions can be used, if the respective ligands are bound to the respective polymers. A polymer of central importance is polyethylene glycol (PEG). This polymer may be easily derivatized with groups like coenzymes, reactive dyes, chelate- and other affinity ligands [163]. The derivatized PEG is able to drag the protein into the polymer-rich phase [164-168].

Another system for liquid phase extraction consists of an aqueous phase in contact with a microemulsion - the latter is also called reverse micellar phase. Microemulsions are thermodynamically stable systems consisting of water, surface active agents and unpolar solvents. Proteins may be solubilized in the so-called reverse micelles. Proteins may be incorporated preferentially into the microemulsion phase by synthesizing surfactants of which the hydrophilic group represents the affinity ligand [169].

The precipitation by means of ammonium sulfate or acetone is a classical process for concentrating and fractionating proteins. A precipitation of higher selectivity and requiring less auxiliary substances may be achieved, if bifunctional ligands or functionalized polymers are used as precipitating agents. Polymers like polyethyleneimine (PEI) or chitosan are well suited in order to precipitate negatively charged proteins. Such polymers may be coupled with affinity ligands like in the case of polyethylene glycol, and may be used for affinity precipitation [166, 170, 171].

Common membrane processes like ultrafiltration are unsuitable for the fractionation of proteins, because of a quite diffuse pore size distribution of membranes and the interaction of different proteins in the stagnant boundary layer [172]. Only if the molar mass of the target protein would be enlarged considerably by artifical means, it could be separated from other proteins present. This may be achieved most simply and selectively by using high molar mass polymers or microscopic supports derivatized with affinity ligands which are effectively retained by porous membranes. When the crude protein mixture is pumped into the ultrafiltration plant, the target protein forms a complex with the support. The complex as such is retained, whereas undesired proteins may leave the ultrafiltration unit. After a washing cycle, the target protein may be liberated by means of the addition of a competing complexing agent. For this purpose, supports have been used in the form of soluble polymers as well as of finely divided porous solid particles [166].

Since the charge and the isoelectric point, respectively, of a protein may be altered quite easily by genetic engineering methods, electrophoretic separation techniques may be of interest, too. For the purpose of production, continuously operating processes would be most appropriate [173]. Respective apparatus with uncompartimented cells are already available [174, 175]. Cells divided by ampholyte membranes and equiped with separate loops for liquid recycling may be used for producing large amounts of proteins discontinuously. Thus, minor differences with respect to the isoelectric point are sufficient in order to obtain proteins of high purity [176].

Survey of Applications

Examples with respect to the use of genetic engineering methods for the improvement of protein separation and purification are gathered in Table 5. Each new issue in the table is initiated by a hyphen. If the hyphen is omitted in one of the columns, the last entry above the actual position is still valid. Some of the signs used in Table 5 have to be explained. The sign "&" signifies that different experiments have been carried out, whereas the sign "+" in the column fusion complement is used when both N- and C-terminal fusions were applied to one target protein.

The entries in Table 5 are arranged according to the kind of interaction. It has to be mentioned that, when protein A (SPA) has been used as a fusion complement, this is to be found under the category of immunoaffinity because it binds to immunoglobulin G (IgG). However, it does not interact with the antigenic determinant, but with the constant F_c-region. This kind of binding is commonly called pseudo-immuno affinity interaction.

With a few exceptions, almost all of the proteins mentioned in Table 5 have been produced by expression in the domestic bug of the molecular geneticists, in *E. coli*. Therefore, the

potential for secretion is quite restricted. By fusion of the target proteins with secretory proteins or signal leader sequences, the respective fusion proteins may be directed into the periplasmic space of *E. coli*. For secretory purposes the genes of e.g. ompA, protein A, protein G or the maltose binding protein have been used for complementation. Eukaryotes and Gram-positive prokaryotes are better suited, if export into the medium is desired. However, protein release into the medium has been observed in rare cases with *E. coli*, too. Otherwise, Table 5 does not contain any indication about signal sequences used for inducing secretion nor about the introduction of special sequences serving as cleavage sites for the fission of fusion proteins. Nevertheless, the respective auxiliaries are indicated which have been used for cleaving the fusion protein - as far as this topic was a subject of investigation. Two cases are included (Table 5, No. 1.10, 2.13) the objective of which has been to obtain fusion proteins of enzymes for facilitating their immobilization. Since the fusion complement serves for binding with the support, the question in this case is similar to that where the objective is downstream processing. However, the very product is the fusion protein exhibiting the activity of the target enzyme.

The β-galactosidase of *E. coli* does not appear particularly noticeable in Table 5, because only recent literature (since ca. 1984) is reviewed. Respective fusion vectors with or without protease cleavage site are commercially available [177]. All fusions with β-galactosidase of *E. coli* are suited *a priori* for the improvement of downstream processing since its substrate affinity for e.g. APTG may be exploited. The compilation of the target proteins clearly shows the great interest for the expression of eukaryotic proteins and particularly those of human origin in rapidly growing prokaryotes. Several of the target proteins mentioned like β-lactamase, SPA-ZZ, β-galactosidase and galactokinase are simply model proteins which have been taken for testing purposes. Table 5 only contains two examples (Table 5, No. 4.11, 7.1) in case of which site-directed mutagenesis has been applied for improving downstream processing. In all other cases purification tags have been introduced by means of fusion.

The ideal fusion complement serving as affinity handle should be able to form a stable complex with an inexpensive, chemically stable and easily derivatized ligand with an absolute specificity. However, the complex should dissociate under mild conditions. In addition, it is desirable that both components affinity handle and ligand would be of simple structure. With respect to these requirements, the FLAG™-peptide may be mentioned. It consists of a sequence of only 8 amino acids (AspTyrLysAsp$_4$Lys-) and may be used for N-terminal fusion. The first four amino acids represent the antigen with the help of which a fusion protein may bind to the monoclonal antibody (mouse anti-FLAG IgG M1) as the ligand. The following sequence (Asp$_3$Lys-) represents the specific binding site for enterokinase which can be used for splitting off the octapeptide from the target protein. Although this case deals with an antibody-antigen complex, dissociation may be achieved under mild conditions because the interaction depends on the presence of calcium ions.

Table 5 : Examples for the improvement of downstream processing of recombinant proteins

Interaction	No.	Example for fusion complement (purification tag)	Example for ligand	Target protein	Host organism	Secr.	Cleavage	References
Immuno affinity	1.1	- FLAG™-peptid	- anti-FLAG mAb	- GM-CSF *etc.*	- *E. coli*	+	- Enterokinase	[189]
	1.1.1				*S. cerevisiae*	ex		
Antibody ⇔ Antigen	1.2			- scFv-PhoA	- *E. coli*	(+)	-	[249]
	1.3			- TNFα, porcine	- *E. coli*	(+)	- Enterokinase	[251]
	1.4	- β-Galactosidase	- anti-β-Gal mAb	- Prolin carrier protein	- *E. coli*	MP	- Collagenase	[190]
"Pseudo-immuno-	1.5			- TRP	- *E. coli*	MP	- Collagenase	[191]
affinity"	1.6		- anti-β-Gal pAb	- hP-Glycoprotein	- *S. cerevisiae*	MP	- Collagenase	[74]
	1.7	- SPA	- IgG	- IGF-I	- *E. coli*	+	- Formic acid	[142, 192,
	1.7.1				*S. aureus*	ex		193]
	1.8			- Lipase	- *S. aureus*	ex	-	[194]
	1.9			- *chain A of ricin*	- *E. coli*	+	- Trypsin	[86]
	1.10			- β-Lactamase	- *E. coli*	+	- EnzImm	[71, 195]
	1.11	- SPA-EE & SPA-ZZ	- IgG	- IGF-I	- *E. coli*	+, ex	- Hydroxylamin	[64, 179]
	1.11.1	SPA-IgG-BD			*S. aureus*	ex		
	1.12	- SPA-ZZ + SPG-B1B2	- IgG or hSA	- IGF-II	- *E. coli*	+	- CNBr	[43]
	1.13	- SPA-ZZ	- IgG	- IGF-II	- *E. coli*	ex	- CNBr	[119]
	1.14	- SPA-DABC		- PRC *subunits*	- *E. coli*	MP	-	[196]
	1.15	- TrpLE-Z	- IgG (not used) chymotrypsin	- BPTI	- *E. coli*	IB	- Chymotrypsin immobilized	[39]

Substrate affinity	2.1	- β-Galactosidase	- APTG	- DNA-BP(ss) & R6K-RI	- *E. coli*	-	- Collagenase	[197-199]
	2.2		- ABTG	- TRP	- *E. coli*	MP	- Collagenase	[191]
Enzyme or enzyme	2.3			- HDNO	- *E. coli*	-	- Factor X_a	[200]
binding site	2.4	- CAT	- *Chloramphenicol*	- *Calcitonin etc.*	- *E. coli*	IB,+	- *Clostripain* &	[201]
⇔							*Staph.* protease	
Substrate, inhibitor or	2.5			- ANF	- *E. coli*	-	- Thrombin etc.	[68, 202]
substrate analogue	2.6	- GST	- Glutathion	- SPA or SPG	- *E. coli*	-	-	[203]
	2.7			- *P. falciparum* Antigens	- *E. coli*	-	- Thrombin, F. X_a	[204]
	2.8			- PTP	- *E. coli*	-	- Thrombin	[85]
	2.9			- CFTR	- *E. coli*	-	-	[224]
	2.10	- ChoBD	- DEAE	- aFGF	- *E. coli*	-	- Factor X_a	[256]
	2.11			- β-Gal	- *E. coli*	-	- Factor X_a	[257]
	2.12	- Glucoamylase SBD	- Starch	- Glucoamylase (partial)	- *E. coli*	-	-	[205]
	2.13	- Exoglucanase CBD	- Cellulose	- β-Glucosidase	- *E. coli*	-	- Enzimm	[206-208]
	2.14	- Cyclodextrin glycosyl-	- α-Cyclodextrin-	- alk. Phosphatase &	- *E. coli*	ex	-	[282]
		transferase	agarose	α-amylase				

General binding	3.1	- β-Galactosidase	- PEG 4000	- SPA or SPG	- *E. coli*	-		[181, 182]
affinity	3.2	- $(AlaTrpTrpPro)_{1-3}$	- PEG 4000	- SPA-ZZ	- *E. coli*	-		[183, 255]
	3.3	- Phosphate-BP	- Hydroxylapatit	- hGHRF	- *E. coli*	+	- CNBr	[117]
Receptor, lectin,	3.4	- Streptavidin	- Biotin	- α-Antitrypsin	- *E. coli*	ex	-	[209]
binding protein	3.5	- SPG & SPG-fragments	- hSA	-	- *E. coli*	+	-	[210]
⇔	3.6	- Maltose-BP	- Starch	- β-Gal & paramyosin	- *E. coli*	+	- Factor X_a	[115, 211]
Binding ligand	3.7			- G5P of phage M13	- *E. coli*	+	-	[105]
(hormone,	3.8			- β-Gal & *PstI*-EN etc.	- *E. coli*	+	-	[115]
saccharide, general	3.9			- SNA & Klenow fragment	- *E. coli*	+	-	[106]
ligand)	3.10			- Leu-zipper fusion	- *E. coli*	+	-	[212]
	3.11			- HIV-1 protease	- *E. coli*	-	- self-cleavage	[80]
	3.12	- CRD	- Galactose	- hPAP	- animal cells	ex	- Trypsin, imm.	[188]
	3.13	- LMM	- (precipitation)	- p21, NF1-GAP, HIV-1 Tat	- E. coli	-	-	[258]
Metal-chelate	4.1	- His_6	- Ni(II)-NTA	- DHFR	- *E. coli*	-	- CP A	[71]
affinity	4.2		- Ni(II)-IDA	- HIV-1 RT	- *E. coli*	-	- hRenin	[83]
	4.3		- Ni(II)-NTA	- wbFos & wbJun	- *E. coli*	IB	-	[213]
(Poly-)histidine	4.4		- Ni(II)-NTA	- HIV-1 RT & -protease	- *E. coli*	-	- HIV-1 protease	[214]
⇔	4.5		- Ni(II)-IDA	- USF	- *E. coli*	-	-	[259]
Metal-chelate	4.6	- His_5 , $ProHis_5Pro$ etc.	- Zn(II)-IDA & Ni(II)-NTA	- F_v-fragment (mouse-IgA)	- *E. coli*	+	-	[47]
	4.7	- HisTrp	- Ni(II)-IDA	- Proinsulin	- *E. coli*	IB	-	[215]
	4.8	- $(His\text{-}X)_n$	- M(II)-IDA	- diverse	- *E. coli*	-	-	[260]
	4.9	- $(AlaHisGlyHisArgPro)_{2-8}$	- Zn(II)- IDA	- SPA-ZZ & β-Gal.	- *E. coli*	+ & -	-	[216]
	4.10	- $(AlaHisGlyHisArgPro)_4$ + + SPG-hSA-BD	- Zn(II)- IDA	- IgG-BD of SPA	- *E. coli*	+	-	[42]
	4.11	- -$His\text{-}X_3\text{-}His$- ; SDM	- Cu(II)-IDA-PEG	- Somatotropin, bovine	-		-	[184]

(Poly-)Arg,Glu,Asp	5.1	- Arg_5	- Kation exchanger	- β-Urogastron	- *E. coli*	-	- CP B	[72, 217,
⇔								218]
Charge ligands	5.2	- Asp_{5-16}	- PEI	- β-Galactosidase	- *E. coli*	-	-	[185, 186]
	5.3	- Glu_n	- Anion exchanger	- hGH	- *E. coli*	-	- DPAP I	[75]
(Poly-)phenylalanine	6.1	- Phe_{11}	- Phenyl-Group	- β-Galactosidase	- *E. coli*	-	-	[219]
⇔								
Hydrophobic ligand								
(Poly-)cysteine	7.1	- Cys (Ser24Cys), SDM	- Thiol-Group	- His64Ala Subtilisin	- *B. subtilis*	ex	-	[220]
⇔	7.2	- Cys_4		- Galactokinase	- *E. coli*	-	-	[219]
Thiol-Group								

Abbreviations :

AB(P)TG = p-aminobenzyl(phenyl)-β-D-thiogalactoside; ANF = atrial natriuratic factor; BD = binding domain; BP = binding protein; BPTI = trypsin inhibitor from bovine pancreas; CAT = chloramphenicol acetyl transferase; CBD = cellulose binding domain; CFTR = cystic fibrosis transmembrane conductance regulator; ChoBD = cholin binding domain of *S. pneumoniae* N-acetylmuramoyl-L-alanine amidase (autolysin); CP = carboxypeptidase; CRD = carbohydrate-recognition domain; DHFR = mouse dihydrofolate reductase; DPAP = dipeptidyl aminopeptidase; EN = endonuclease; EnzImm = fusion aiming at immobilization of the target enzyme; ex = export into the medium; aFGF = acidic fibroblast growth factor; GH = growth hormone; GHRF = growth hormone release factor; GM-CSF = colony stimulating factor for granulocytes and macrophages; GST = glutathion-S transferase; G5P = gene-5 protein, binds single-strand DNA; h.. = human-; HDNO = 6-hydroxy-D-nicotin oxidase; IB = inclusion bodies; IDA = iminodiacetic acid; IGF = insulin-like growth factor; IgG = immunoglobulin G; IL-2 = Interleukin 2; LMM = C-terminal fragment of light meromyosin from rabbit; mAb = monoclonal antibody; MP = membrane protein; NTA = Nitrilotriacetic acid; pAB = polyclonal antibody; PAP = placenta alkaline phosphatase; PEG = Polyethylene glycol; PhoS = phosphate binding protein; RT = reverse transcriptase; R6K-RI = RK6 replication initiator; PRC = photosynthetic reaction centre (from *R. sphaeroides*); PTP = protein tyrosine phosphatase from rat brain; SA = serum albumin; SBD = starch binding domain; scFv = single-chain antigen binding protein (variable mAb-domain for recognition of human erbB-2 protein); SDM = site-directed mutagenesis; SNA = *Staphylococcus aureus* nuclease A; SPA = *Staphylococcus* protein A; SPA-EE(ZZ) = dimer of a (synthetic) IgG binding fragment of SPA; SPG = *Staphylococcus* protein G; SPG-B1B2 = fusion of two hSA binding domains from SPG; TNFα = porcine tumor necrosis factor alpha; TRP = tetracycline resistance protein (*tet*); ss = single strand-; TrpLE = leader sequence of the *trp*-operon; USF = human gene-specific transcription factor; Z = synthetic IgG binding domain of SPA.

The fusion protein is only bound in the presence of calcium ions, whereas the complex dissociates when calcium ions are withdrawn. Since the FLAG-peptide is small and very hydrophilic, it can be expected that it may be found at the protein surface and thus may be accessible [178]. The dependence on the availability of specific antibodies, however, has to be taken into account for an economic analysis of the separation process.

Fusion complements based on protein A from *Staphylococcus aureus* are of interest in many respects. This protein is stable against proteolysis and forms stable complexes with the constant region (F_C) of IgG of different mammals. The wild type gene consists of a signal leader sequence for secretion, five IgG-binding domains (E,D,A,B,C) and an additional binding domain for the integration of protein A in the cell wall of *S. aureus*. Fusion complements may be selectively assembled by taking e.g. the signal sequence together with a single or a repeated sequence of binding domains [142]. The complement SPA-ZZ is such a designed complement consisting of the signal leader sequence and two B-domains containing two point mutations. These point mutations (AsnGly→AsnAla) were introduced in order to protect the B-domain against hydrolysis by hydroxylamine. A site directed mutation at the N-terminus of e.g. IGF-I (Met→Asn) is sufficient for the introduction of a cleavage side (Asn↓Gly) to allow the fusion protein to be split by means of hydroxylamin. By using both fusion complements SPA-EE and -ZZ it has even been observed that about 80% of the fusion protein with IGF-I or -II expressed in *E. coli* were exported into the medium [64, 119]. Fermentation capacities up to 1000 L have already been applied for the production of IGF-I [179]. Vectors for the assembly of SPA-fusions are commercially available [177]. Plasmids with a temperature - inducible promoter have been described [252] as well as one which permits a C-terminal fusion with SPA-ZZ [253].

The periplasmic maltose binding protein of *E. coli* is another commonly used fusion complement. It may be used for inducing the secretion into the periplasm. The fusion product adsorbs on cross linked starch, whereas elution may be achieved in the presence of maltose. A disadvantage of this purification tag is its relatively high molar mass (about 41 kDa) in comparison with e.g. SPA-ZZ (about 14 kDa) or the FLAG-peptide (< 1 kDa). However, an advantage which should not be underestimated is given by the fact that an inexpensive ligand can be used.

An inexpensive ligand is also used in the case of the metal-chelate technique. The complementation of the target protein with histidine-rich sequences can easily be achieved. With the introduction of vicinal histidine residues and their interaction with Ni(II)- or Zn(II)-activated chelating groups, a high selectivity may be obtained [254]. Some cloning kits are commercially available which are based on a His_6 fusion complement [180].

A categorization according to the kind of separation or purification process is not given in Table 5. This is due to the fact that other processes than adsorption and chromatography,

respectively, have rarely been applied. Extraction in aqueous two-phase systems may successfully be used, if the fusion complement interacts specifically with the soluble polymer of the phase system (e.g. PEG). It is known that β-galactosidase of *E. coli* is characterized by an exceptionally high distribution coefficient in favour of the PEG-rich phase of PEG-salt systems. If e.g. protein A or G are linked with β-galactosidase, an essentially more favourable distribution may be found compared with the extraction of the original proteins [181, 182]. Tryptophan side chains on the surface of β-galactosidase are thought to be responsible for the favourable partitioning of this enzyme. This assumption has impressively been proved by the design of a fusion complement consisting of the peptide AlaTrpTrpPro ("partitioning peptide") and the use of oligomers of this sequence [183,255]. In such cases specific affinity ligands are not required. However, the interaction with specific affinity ligands bound to a polymer of the aqueous two-phase system is of more general application. The influence of X→His point mutations on the affinity partitioning in Co(II)-IDA-PEG-doted PEG-salt systems has been studied. If histidine residues are introduced into the protein backbone in such a way that two of them may be found in vicinal position on an α-helical structure (His-X_3-His) on the protein surface, a considerable improvement of the distribution in favour of the polymer-rich phase can be expected [184].

Only two groups of authors report about the application of affinity precipitation. Fusion proteins of β-galactosidase of *E. coli* with polyaspartate sequences of various length have been precipitated by means of polyethyleneimine [185, 186]. Relatively long polyaspartate sequences were required in order to achieve an appreciable accumulation of precipitate - after nucleic acids had been broken down [185]. Ligands with more selective interaction would certainly be better suited as precipitating agents [e.g. 187]. A fusion protein of human P-glycoprotein and β-galactosidase has been recovered by immunoprecipitation [74]. In this case the fusion protein was complexed with polyclonal antibodies against β-galactosidase of rabbit and was precipitated with protein A bound to sepharose which means that the fusion protein was recovered adsorbed to the sepharose particles. Auto-precipitation may be induced by utilizing the C-terminal fragment of light meromyosin (LMM) from rabbit fast skeletal muscle as the fusion complement. The myosin heavy chains undergo coiled-coil interaction. Such fusion proteins precipitate in dilute salt solutions whilst they are soluble in concentrated salt solutions [258].

If the fusion protein is cleaved in order to obtain the target protein in its native form, the fusion complement may be removed by means of the same strategy used for the separation of the fusion protein [18]. This principle confers a certain charme to the strategy of fusion. One problem remains to be solved, that is the removal of the protease used for cleaving the fusion product. The immobilization of proteases on macroporous supports of small particle size might eventually solve this problem in an economic manner [188, 235].

PROSPECT

This short review under the subject of genetic engineering methods for improvement of downstream processing of proteins has shown that extremely interesting strategies have been developed which are equally being applied in both areas research and production. However, It has to be mentioned that still a lot of problems have to be solved. The practical application of these strategies still holds many surprises due to superficial knowledge about protein expression, folding, sorting, and post-translational processing. The safe knowledge accumulated so far together with the rapid progression in this area, however, will certainly lead to a broad application of the strategies briefly outlined here for the production of recombinant proteins.

REFERENCES

1. Bayer, E.: Towards the Chemical Synthesis of Proteins, Angew. Chem. - Int. Ed. 30 (2) (1991) 113-129
2. Onken, U.: Physikalisch-chemische Trennverfahren in der Biotechnologie, Chem.-Ing.-Tech. 61 (5) (1989) 395-402
3. Wang, D.I.C.: Biotechnology: status and perspectives, AIChE Monograph Ser. 84 (18) (1988) 1-22
4. Ogez, J.R., Hodgdon, J.C., Beal, M.P., Builder, S.E.: Downstream Processing of Proteins: Recent Advances, Biotech. Adv. 7 (1989) 467-488
5. Gordon, N.F., Moore, C.M.V., Cooney, C.L.: An Overview of Continuous Protein Purification Processes, Biotech. Adv. 8 (1990) 741-762
6. Lee, S.-M.: The Primary Stages of Protein Recovery, J. Biotechnol. 11 (1989) 103-118
7. Hammond, P.M., Scawen, M.D.: High-Resolution Fractionation of Proteins in Downstream Processing, J. Biotechnol. 11 (1989) 119-134
8. Harris, E.L.V., Angal, S. (Eds.): Protein Purification Methods: A Practical Approach, IRL Press, Oxford 1989
9. Harris, E.L.V., Angal, S. (Eds..): Protein Purification Applications: A Practical Approach, IRL Press, Oxford 1989
10. Janson, J.C., Ryden, L.G. (Eds.): Protein Purification, VCH Verlagsgesellschaft, Weinheim - New York 1989
11. Verrall, M.S., Hudson, M.J. (Eds..): Separations for Biotechnology, Ellis Horwood/Halsted Press, 1987
12. Deutscher, M.P. (Ed.): Guide to Protein Purification, Methods Enzymol. 182, Academic Press, San Diego - London 1990
13. Ladisch, M. (Ed.): Protein Purification, ACS Symp. Ser. 427, VCH/ACS, Weinheim - New York 1990

14. Hamel, J.-F.P., Hunter, J.B. (Eds.): Downstream Processing and Bioseparation, ACS Symp. Ser. 419, VCH/ACS, Weinheim - New York 1990
15. Fisher, D., Sutherland, I. (Eds.): Separations Using Aqueous Phase Systems, Plenum Publishing, New York - London 1989
16. Sassenfeld, H.M.: Engineering Proteins for Purification, Trends Biotechnol. 8 (1990) 88-93
17. Hammond, P.M., Atkinson, T., Sherwood, R.F., Scawen, M.D.: Manufacturing New-Generation Proteins: Part I + II, Pharm. Tech. Int. (April 1991) 24-32, (May 1991) 16-19
18. Uhlén, M., Moks, T.: Gene Fusions for Purpose of Expression: An Introduction, Methods Enzymol. 185 (1990) 129-143
19. Sherwood, R.: Protein Fusions: Bioseparation and Application, Trends Biotechnol. 9 (1991) 1-3
20. Summers, K.: The Kinetics of Plasmid Loss, Trends Biotechnol. 9 (8) (1991) 73-278
21. Kumar, P.K.R., Maschke, H.-E., Friehs, K., Schügerl, K.: Strategies for Improving Plasmid Stability in Genetically Modified Bacteria in Bioreactors, Trends Biotechnol. 9 (8) (1991) 279-284
22. Nordström, K., Uhlin, B.E.: Runaway-replication plasmids as tools to produce large quantities of proteins from cloned genes in bacteria, Bio/Technol. 10 (1992) 661-666
23. Makoff, A.J., Oxer, M.D.: High level heterologous expression in *E. coli* using mutant forms of the *lac* promoter, Nucl. Acids Res. 19 (1991) 2417-2421
24. Yasukawa, K., Saito, T.: A new system for the expression of human interleukin-6 in *Escherichia coli*, Biotech. Lett. 12 (1990) 419-424
25. Elvin, C.M., Thompson, P.R., Argall, M.E., Hendry, P., Stamford, N.P.J.: Modified Bacteriophage-Lambda Promoter Vectors for Overproduction of Proteins in *Escherichia coli*, Gene 87 (1990) 123-126
26. Dikshit, K.L., Dikshit, R.P., Webster, D.A.: Study of Vitreoscilla Globin (Vgb) Gene-Expression and Promoter Activity in *Escherichia coli* Through Transcriptional Fusion, Nucl. Acids Res. 18 (1990) 4149-4155
27. Pharmacia P-L Biochemicals, Inc.: Molecular and Cell Biology Cataloque 1991/1992
28. Brawerman, G.: Mechanism of mRNA decay, Trends Biotechnol. 8 (1990) 171-174
29. Chan, W.K.Y., Belfort, G., Belfort, M.: Protein overproduction in *Escherichia coli*: RNA stabilization, cell disruption and recovery with a cross-flow microfiltration membrane, J. Biotechnol. 18 (1991) 225-242
30. Schmucker, R., Gülland, U., Will, M., Hillen W.: High level expression of *Acinetobacter calcoaceticus* mutarotase in *Escherichia coli* is achieved by improving the translational control sequence and removal of the signal sequence, Appl. Microb. Biotechnol. 30 (1989) 509-514
31. Olsen, M.K., Rockenbach, S.K., Curry, K.A., Tomich, C.-S.C.: Enhancement of heterologous polypeptide expression by alterations in the ribosome-binding-site sequence, J. Biotechnol. 9, (1989) 179-190

32. Surek, B., Wilhelm, M., Hillen, W.: Optimizing the Promoter and Ribosome Binding Sequence for Expression of Human Single Chain Urokinase-Like Plasminogen-Activator in *Escherichia coli* and Stabilization of the Product by Avoiding Heat-Shock Response, Appl. Microb. Biotechnol. 34, (1991) 488-494
33. Spanjaard, R.A., van Dijk, M.C.M., Turion, A.J., van Duin J.: Expression of the rat interferon-α_1 gene in *Escherichia coli* controlled by the secondary structure of the translation-initiation region, Gene 80, (1989) 345-351
34. Ernst, J.F.: Codon usage and gene expression, Trends Biotechnol. 6, (1988) 196-199
35. Gottesman, S.: Minimizing Proteolysis in *Escherichia coli*: Genetic Solutions, Methods Enzymol. 185 (1990) 119-129
36. Gottesman, S.: Genetics of proteolysis in *Escherichia coli*, Ann. Rev. Genet. 23 (1989)163-198
37. Friehs, K., Bailey, J.E.: Unusual observation during construction of a new cloning vector providing *lon* gene expression in *Escherichia coli*, J. Biotechnol. 9 (1989) 305-316
38. Brill, J.A., Quinlane-Walshe, C., Gottesman, S.: Fine-Structure Mapping and Identification of Two Regulators of Capsule Synthesis in *Escherichia coli* K-12, J. Bacteriol. 170 (1988) 2599-2611
39. Altmann, J.D., Henner, D., Nilsson, B., Anderson, S., Kuntz, I.D.: Intracellular Expression of BPTI Fusion Proteins and Single Column Cleavage/Affinity Purification by Chymotrypsin, Protein Eng. 4 (5) (1991) 593-600
40. Cao, J., Revzin, A., Ferguson-Miller, S.: Conversion of a Mitochondrial Gene for Mamalian Cytochrome c Oxidase Subunit II into its Universial Codon Equivalent and Expression *in vivo* and *in vitro*, Biochem. 30 (1991) 2642-2650
41. Hellebust, H., Uhlén, M., Enfors, S.-O.: Effect of protein fusion on the stability of proteolytically sensitive sites in recombinant DNA proteins, J. Biotechnol. 12 (1989) 275-284
42. Jansson, B., Palmcrantz, C., Uhlén, M., Nilsson, B.: A Dual-Affinity Gene Fusion System to Express Small Recombinant Proteins in a Soluble Form: Expression and Characterization of Protein A Deletion Mutants, Prot. Eng. 2 (6) (1989) 555-561
43. Hammarberg, B., Nygren, P.-A., Holmgren, E., Elmblad, A., Tally, M., Hellman U., Moks, T., Uhlén, M.: Dual Affinity Fusion Approach and Its Use to Express Recombinant Human Insulin-Like Growth Factor II, Proc. Natl. Acad. Sci. USA 86 (1989) 4367-4371
44. Baneyx, F., Ayling, A., Palumbo, T., Thomas, D., Georgiou G.: Optimization of Growth Conditions for the Production of Proteolytically-Sensitive Proteins in the Periplasmatic Space of *Escherichia coli*, Appl. Microb. Biotechnol. 36 (1991) 14-20
45. Farrell, D.J.: Purification of Recombinant Proteins for Pharmaceutical Use, Biochem. Soc. Trans. 18 (1990) 243-245

46. Bialy, H.: Recombinant Proteins: Virtual Authenticity, Bio/Technology 5 (1987) 883-890
47. Skerra, A., Pfitzinger, I., Plückthuhn, A.: The Functional Expression of Antibody F_V Fragments in *Escherichia coli*: Improved Vectors and a Generally Applicable Purification Technique, Bio/Technology 9 (1991) 273-278
48. Wilkinson, D.L., Harrison, R.G.: Predicting the Solubility of Recombinant Proteins in *Escherichia coli*, Bio/Technology 9 (1991) 443-448
49. Krueger, J.K., Kulke, M.N., Schutt, C., Stock,J.: Protein Inclusion Body Formation and Purification, BioPharm 2 (1989) 40-47
50. Forman, S.M., DeBernardez, E.R., Feldberg, R.S., Swartz, R.W.: Crossflow Filtration for the Separation of Inclusion Bodies from Soluble Proteins in Recombinant *Escherichia coli* Cell lysate, J. Membrane Sci. 48 (1990) 263-279
51. Thatcher, D.R.: Recovery of Therapeutic Proteins from Inclusion Bodies: Problems and Process Strategies, Biochem. Soc. Trans. 18 (1990) 234-235
52. Schein, C.H.: Solubility as a function of protein structure and solvent components, Bio/Technology 8 (1990) 308-315
53. Strandberg, L., Enfors S.-O.: Factors Influencing Inclusion Body Formation in the Production of a Fused Protein in *Escherichia coli*, Appl. Env. Microb. 57 (1991) 1669-1674
54. Kotewicz, M.L., D'Alessio, J.M., Driftmeier, K.M., Blodgett, K.P., Gerard, G.F.: Cloning and overexpression of Moloney murine leukemia virus reverse transcriptase in *Escherichia coli*, Gene 35 (1985) 249-258
55. Titchener-Hooker, N.J., Gritsis, D., Olbrich R., Mannweiler, K., Gardiner, S.A.M., Fish, N.M., Hoare, M.: Integrated Process Design for Producing and Recovering Proteins from Inclusion Bodies, Pharm. Tech. Int. (Oct. 1990) 42-48
56. Fischer, G., Schmid, F.X.: The Mechanism of Protein Folding. Implications of *in vitro* Refolding Models for *de novo* Protein Folding and Translocation in the Cell, Biochem. 29 (1990) 2205-2212
57. Dill, K.A.: Dominant Forces in Protein Folding, Biochem. 29 (1990) 7133-7155
58. Jaenicke, R.: Folding and Association of Proteins, Progr. Biophys. Mol. Biol. 49 (1987) 117-237
59. Spalding, B.J.: Downstream Processing: Key to Slashing Production Costs 100 Fold, Bio/Technology 9 (1991) 229-234
60. Cleland, J.L., Builder, S.E., Swartz, J.R., Winkler, M., Chang, J.Y., Wang, D.I.C.: Polyethylene glycol enhanced protein folding, Bio/Technol. 10 (1992) 1013-1019
61. Gatenby, A.A., Viitanen, P.V., Lorimer, G.H.: Chaperonin assisted polypeptide folding and assembly: implication for the production of functional proteins in bacteria, Trends Biotechnol. 8 (1990) 354-358
62. Horwich, A.L., Neupert, W., Hartl F.-U.: Protein-catalysed protein folding, Trends Biotechnol. 8 (1990) 126-131

63. Carter, P.: Side-Specific Proteolysis of Fusion Proteins, ACS Symp. Ser. 427 (1990) 181-193

64. Moks, T., Abrahmsén, L., Holmgren, E., Bilich, M., Olsson, A., Uhlén, M., Pohl, G., Sterky, C., Hultberg, H., Josephson, S., Holmgren, A., Jörnvall, H., Nilsson, B.: Expression of Human Insulin-Like Growth Factor I in Bacteria: Use of Optimized Gene Fusion Vectors to Facilitate Protein Purification, Biochem. 26 (1987) 5239-5244

65. Szoka, P.R., Schreiber, A.B., Chan ,H., Murthy, J.: A General Method for Retrieving the Components of a Genetically Engineered Fusion Protein, DNA 5 (1986) 11-15

66. Huston, J.S., Levinson, D., Mudgett-Hunter, M., Tai, M.-S., Novotny, J., Margolies, M.N., Ridge, R.J, Bruccoleri, R.E., Haber, E., Crea, R., Oppermann, H.: Protein engineering of antibody binding sites: Recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*, Proc. Nat. Acad. Sci. USA 85 (1988) 5879-5883

67. Hüsken, D., Beckers, T., Engels, J.W.: Overexpression in *Escherichia coli* of a Methionine-Free Designed Intereukin-2 Receptor (Tac Protein) Based on a Chemically Cleavable Fusion Protein, Eur. J. Biochem. 193 (1990) 387-394

68. Knott, J.A., Sullivan, C.A., Weston, A.: The Isolation and Characterization of Human Atrial Natriuratic Factor Produced as a Fusion Protein in *Escherichia coli*, Eur. J. Biochem. 174 (1988) 405-410

69. Villa, S., De Fazio, G., Donini, S., Tarchi, G., Canosi, U.: Expression in *Escherichia coli* and characterization of human growth-hormone-releasing factor, Eur. J. Biochem 171 (1988) 137-141

70. Shechter, Y., Patchornik, A., Burstein, Y.: Selective Chemical Cleavage of Tryptophanyl Peptide Bonds by Oxidative Chlorination with *N*-Chlorosuccinimide, Biochem. 15 (1976) 5071-5075

71. Hochuli, E., Bannwarth, W., Döbeli, H., Gentz, R., Stüber, D.: Genetic Approach to Facilitate Purification of Recombinant Proteins with a Novel Metal Chelate Adsorbent, Bio/Technology 11 (1988) 1321-1325

72. Smith, J.C., Derbyshire, R.B., Cook, E., Dunthorne, L., Viney, J., Brewer, S.J., Sassenfeld, H.M., Bell, L.D.: Chemical Synthesis and Cloning of a Poly(arginin)-Coding Gene Fragment Designed to Aid Polypeptide Purification, Gene 32 (1984) 321-327

73. Dahlman, K., Strömstedt, P.-E., Rae, C., Jörnvall, H., Flock, J.-I., Carlstedt-Duke, J., Gustafsson, J.-A.: High Level Expression in *Escherichia coli* of the DNA-binding Domain of the Glucocorticoid Receptor in a Functional Form Utilizing Domain-specific Cleavage of a Fusion Protein, J. Biol. Chem. 264 (1989) 804-809

74. Shimabuku, A.M., Saeki, T., Ueda, K., Komano, T.: Production of a Side Specifically Cleavable P-Glycoprotein-β-Galactosidase Fusion Protein, Agric. Biol. Chem. 55 (4) (1991) 1075-1080

75. Dalbøge, H., Dahl, H.-H.M., Pedersen, J., Hansen, J.W., Christensen, T.: A Novel Enzymatic Method for Production of Authentic hGH from an *Escherichia coli* Produced hGH-Precurser, Bio/Technology 5 (2) (1987) 161-164

76. Allen, G, Payntner, C.A., Winther, M.D.: Production of epidermal growth factor in *Escherichia coli* from a synthetic gene, J. Cell Sci. Suppl. 3 (1985) 29-37

77. Prickett, K.S., Amberg, D.C., Hopp, T.P.: A Calcium-Dependent Antibody for Identification and Purification of Recombinant Proteins, BioTechniques 7 (1989) 580-589

78. McPherson, D.T., Morrow, C., Minehan, D.S., Wu, J., Hunter, E., Urry, D.W.: Production and purification of a recombinant elastomeric polypeptide, G-$(VPGVG)_{19}$-VPGV, from *Escherichia coli*, Biotechnol. Prog. 8 (1992) 347-352

79. Montgomery, D.S., Singh, O.K., Gray, N.M., Dykes, C.W., Weir, M.P., Hobden, A.N.: Expression of an Autoprocessing CAT-HIV-1 Proteinase Fusion Protein: Purification to Homogeneity of the Released 99 Residue Proteinase, Biochem. Biophys. Res. Comm. 175 (1991) 784-794

80. Louis, J.M., McDonald, R.A., Nashed, N.T., Wondrak, E.M., Jerina, D.M., Oroszlan, S., Mora, P.T.: Autoprocessing of the HIV-1 Protease Using Purified Wild-Type and Mutated Fusion Proteins Expressed at High Levels in *Escherichia coli*, Eur. J. Biochem. 199 (2) (1991) 361-369

81. Tonouchi, N., Oouchi, N., Kashima, N., Kawai, M., Nagase, K., Okano, A., Matsui, H., Yamada K., Hirano, T., Kishimoto, T.: High-Level Expression of Human BSF-2/IL-6 cDNA in *Escherichia coli* Using a New Type of Expression-Preparation System, J. Biochem. 104 (1988) 30-43

82. Takahara, M., Sagai, H., Inouye, S., Inouye, M.: Secretion of Human Superoxide Dismutase in *Escherichia coli*, Bio/Technology 6 (1988) 195-198

83. Sharma, S.K., Evans, D.B., Vosters, A.F., McQuade, T.J., Tarpley, W.G.: Metal Affinity Chromatography of Recombinant HIV-1 Reverse Transcriptase Containing a Human Renin Cleavable Metal Binding Domain, Biotech. Appl. Biochem. 14 (1991) 69-81

84. Carter, P., Nilsson, B., Burdick, D., Wells J.A.: Engineering Subtilisin BPN' for Site-Specific Proteolysis, Proteins: Structure, Function and Genetics 6 (1989) 240

85. Hakes, D.J., Dixon, J.E.: New vectors for high level expression of recombinant proteins in bacteria, Anal. Biochem. 202 (1992) 293-298

86. O'Hare, M., Brown, A.N., Hussain, K., Gebhardt, A., Watson, G., Roberts, L.M., Vitetta, E.S., Thorpe, P.E., Lord, J.M.: Cytotoxicity of a Recombinant Ricin-A-Chain Fusion Protein Containing a Proteolytically-Cleavable Spacer Sequence, FEBS Lett. 273 (1) (1990) 200-204

87. Miller, H.I., Henzel, W.J., Ridgway, J.B., Kuang, W.-J., Chisholm, V., Liu, C.-C.: Cloning and expression of a yeast ubiquitin-protein cleaving activity in *Escherichia coli*, Bio/Technology 7 (1989) 698-704

88. Ogden, K.L., Taylor, A.L.: Genetic control of flocculation in *Escherichia coli*, J. Ind. Microbiol. 7 (1991) 279-286
89. Harrison, S.T.L.: Bacterial cell disruption: A key unit operation in the recovery of intracellular products, Biotech. Adv. 9 (1991) 217-240
90. Scawen, M.D., Hammond, P.M., Sherwood, R.F., Atkinson, T.: Large-Scale Protein Extraction and Isolation, Biol. Soc. Trans. 18 (2) (1990) 231-233
91. Kula, M.R., Schütte, H.: Purification of Proteins and the Disruption of Microbial Cells, Biotechnol. Progr. 3 (1987)131-142
92. Dabora, R.L., Cooney, C.L.: Intracellular Lytic Enzyme systems and their use for disruption of *Escherichia coli*, Adv. Biochem. Eng. /Biotechnol. 43 (1990) 11-30
93. US 4,948,735, 14.Aug.1991, Repligen Corp., (Luria S.E., Suit J.L., Jackson J.A.: System for Release of Proteins from Microbe Cells)
94. Dabora, R.L., Eberiel, D.T., Cooney, C.L.: Release of β-galactosidase from *E. coli* by a plasmid containing a temperature sensitive lytic function, Biotech. Lett. 11 (1989) 845-850
95. Nicaud, J.-M., Mackman, N., Holland, I.B.: Current status of secretion of foreign proteins by microorganisms, J. Biotechnol. 3 (1986) 255-270
96. Rasched, I., Oberer-Bley, E.: *Escherichia coli* und die Biotechnologie - ein ideales Paar, Chem. Lab. Biotech. 41 (1990) 36-42
97. Hsiung, M.H., Becker, G.W.: Secretion and folding of human growth hormone in *Escherichia coli*, Biotechnol. Gen. Eng. Rev. 6 (1988) 43-65
98. Lee, C., Li, P., Inouye, H., Brickman, E.R., Beckwith, J.: Genetic Studies on the Inability of β-Galactosidase to be Translocated across the *Escherichia coli* Cytoplasmic Membrane, J. Bacteriol. 171 (1989) 4609-4616
99. Alexander, P., Oriel, P.J., Glassner, D.A., Grulke, E.A.: Continuous culture of *Escherichia coli* for extracellular production of recombinant amylase, Biotechnol. Lett. 11 (1989), 609-614
100. Emerick, A.W., Bertolani, B.L., Ben-Bassat, A., White, T.J., Konrad, M.W.: Expression of a *Escherichia coli* preproinsulin fusion protein in *Escherichia coli*, Bio/Technology 2 (1984) 165-168
101. Zemel-Dreasen, O., Zamir, A.: Secretion and processing of an immunoglobulin light chain in *Escherichia coli*, Gene 27 (1984) 315-322
102. Georgiou, G., Shuler, M.L., Wilson, D.B.: Release of Periplasmic Enzymes and Other Physiological Effects of β-Lactamase Overproduction in *Escherichia coli*, Biotechnol. Bioeng. 32 (1988) 741-748
103. Ohgai, H., Kumakura, T., Komoto S., Matsuo, Y., Oshiden, K., Koide, T., Yanaihara, C., Yanaihara, N.: Production of rat epidermal growth factor by *Escherichia coli* cells containing a secretion plasmid, J. Biotechnol. 10 (1989) 151-160
104. Summers, R.G., Knowles, J.R.: Illicid Secretion of a Cytoplasmic Protein into the Periplasm of *Escherichia coli* Requires a Signal Peptide Plus a Portion of the Cognate Secreted Protein, J. Biol. Chem. 264 (1989) 20074-20081

105. Blondel, A., Bedouelle, H.: Export and Purification of a Cytoplasmic Dimeric Protein by Fusion to the Maltose-Binding Protein of *Escherichia coli*, Eur. J. Biochem. 193 (1990) 325-330

106. Bedouelle, H., Duplay, P.: Production in *Escherichia coli* and One-Step Purification of Bifunctional Hybrid Proteins which Bind Maltose - Export of the Klenow Polymerase into the Periplasmic Space, Eur. J. Biochem. 171 (1988) 541-549

107. Libby, R.T., Braedt, G., Kronheim, S.R., March, C.J., Urdal, D.L., Chiaverotti, T.A., Tushinski, R.J., Mochizuki, D.J., Hopp, T.P., Cosman, D.: Expression and Purification of Native Human Granulocyte-Macrophage Colony-Stimulating Factor from an *Escherichia coli* Secretion Vector, DNA 6 (1987) 221-229

108. Barbero, J.O.L., Buesa, J.M., Penalva, M.A., Perez-Aranda, A., Garcia, J.L.: Secretion of mature human interferon alpha 2 into the periplasmic space of *Escherichia coli*, J. Biotechnol. 4 (1986) 255-267

109. Habuka, N., Akiyama K., Hideaki, T., Miyano, M., Matsumoto, T., Noma, M.: Expression and Secretion of *Mirabilis* Antiviral Protein in *Escherichia coli* and Its Inhibition of *in vitro* Eukaryotic and Prokaryotic Protein Synthesis, J. Biol. Chem. 265 (19) (1990) 10988-10992

110. Henze, P.-P.C., Hahn, U., Erdmann, V.A., Ulbrich, N.: Expression of the chemically synthesized coding region for the cytotoxin α-sarcin in *Escherichia coli* using a secretion cloning vector, Eur. J. Biochem. 192 (1990) 127-131

111. Shibui, T., Matsui, R., Uchida-Kamizono, M., Okazaki, H., Kondo, J., Nagahari, K., Nakanishi, S., Teranishi, Y.: Periplasmic production of human pancreatic prokallikrein in *Escherichia coli*, Appl. Microb. Biotechnol. 31 (1989) 253-258

112. Marks, C.B., Vasser, M., Ng, P., Henzel, W., Anderson S.: J. Biol. Chem. 261 (1986) 7115-7118

113. Oka, T., Sumi, S., Fuwa, T., Yoda, K., Yamasaki, M., Tamura, G., Miyake, T.: Efficacies of Different Secretion Vectors for Secretion of Human Epidermal Growth Factor by *Escherichia coli*, Agric. Biol. Chem. 51 (4) (1987) 1099-1104

114. Ohsuye, K., Nomura, M., Tanaka, S., Kubota, I., Nakazato, H., Shinagawa, H., Nakata, A., Noguchi, T.: Expression of chemically synthesized α-neo-endorphin gene fused to *E. coli* alkaline phosphatase, Nucl. Acids Res. 11 (5) (1983) 1283-1294

115. di Guan, C., Li, P., Riggs, P.D., Inouye, H.: Vectors that Facilitate the Expression and Purification of Foreign Peptides in *Escherichia coli* by Fusion to Maltose-Binding Protein, Gene 67 (1988) 21-30

116. Fujimura, T., Tanaka, T., Kanako, O., Morioka, H., Uesugi, S., Ikehara, M., Nishikawa S.: Secretion of recombinant ribonuclease T_1 into the periplasmic space of *Escherichia coli* with the aid of the signal peptide of alkaline phosphatase, FEBS 269 (1,2) (1990) 71-74

117. Anba, J., Baty, D., Lloubès, R., Pagès, J.-M., Joseph-Liauzun, E., Shire, D., Roskam, W., Lazdunski, C.: Expression Vector Promoting the Synthesis and Export of the Human Growth-Hormone-Releasing Factor in *Escherichia coli,* Gene 53 (1987) 219-226

118. Hogset, A., Blinsmo, O.R., Saether, O., Gautvik, V.T., Holmgren, E., Hartmanis, M., Josephson, S., Gabrielsen, O.S., Gordelaze, J.O., Alestrom, P., Gautvik, K.M.: Expression and Characterization of a Recombinant Human Parathyroid Hormone Secreted by *Escherichia coli* Empolying the Staphylococcal Protein A Promoter and Signal Sequence, J. Biol. Chem. 265 (13) (1990) 7338-7344

119. Wadenstein, H., Ekebacke, A., Hammarberg, B., Holmgren, E., Kalderen, C., Tally, M., Moks, T., Uhlén, M., Josephson, S., Hartmanis, M.: Purification and Characterization of Recombinant Human Insulin-Like Growth Factor II (IGF-II) Expressed as a Secreted Fusion Protein in *Escherichia coli*, Biotech. Appl. Biochem. 13 (3) (1991) 412-421

120. Fraser, T.H., Bruce, T.J.: Chicken ovalbumin is synthesized and secreted by *Escherichia coli*, Proc. Natl. Acad. Sci. USA 75 (12) (1978) 5936-5940

121. Kornacker, M.G., Pugsley, A.P.: The Normally Periplasmic Enzyme β-Lactamase is Specifically and Efficiently Translocated Through the *Escherichia coli* Outer Membrane when it is Fused to the Cell-Surface Enzyme Pullulanase, Mol. Microbiol. 4 (1990) 1101-1109

122. Talmadge, K., Brosius, J., Gilbert, W.: An 'internal' signal sequence directs secretion and processing of proinsulin in bacteria, Nature 294 (1981) 176-178

123. Morioka-Fujimoto, K, Marumoto, R., Fukuda, T.: Modified Enterotoxin Signal Sequences Increase Secretion Level of the Recombinant Human Epidermal Growth Factor in *Escherichia coli*, J. Biol. Chem. 266 (3) (1991) 1728-1732

124. US 4,945,047, 31. Jul. 1991, Sanofi S.A. (Legoux R., Leptlatois P., Joseph-Liauzun, E.)

125. Letoffe, S., Delepelaire, P., Wandersman, C.: Cloning and Expression in *Escherichia coli* of the *Serratia marcescens* Metalloprotease Gene: Secretion of the Protease from *E. coli* in the Presence of the *Erwinia chrysanthemi* Protease Secretion Functions, J. Bacteriol. 173 (7) (1991) 2160-2166

126. van Putten, A.J., Graaf, de F.K., Oudega, B.: Engineering Protein Export in *Escherichia coli*: The Cloacine DF13 System, Eur. Conf. Biotechnol. 4 (1987) 593-601

127. Mackman, N., Nicaud, J.M., Gray, L., Holland, I.B.: Secretion of Hemolysin by *Escherichia coli*, Curr. Top. Microbiol. Immunol. 125 (1986) 159-181

128. Payne, M.S., Jackson, E.N.: Use of Alkaline Phosphatase Fusions To Study Protein Secretion in *Bacillus subtilis*, J. Bacteriol. 173 (7) (1991) 2278-2282

129. Hemilä, H., Sibakov, M.: Production of Heterologous Proteins in *Bacillus subtilis*: The Effect of the Joint between Signal Sequence and Major Protein on Yield, Appl. Microb. Biotechnol. 36 (1991) 61-64

130. Honjo, M., Akaoka, A., Nakayama, A., Furutani, Y.: Secretion of human growth hormone in *Bacillus subtilis* using prepropeptide coding region of *Bacillus amyloliquefaciens* neutral protease gene, J. Biotechnol. 4 (1986) 63-71
131. Carter, J.J., Yaegashi, N., Jenison, S.A., Galloway, D.A.: Expression of Human Papyloma Virus Proteins in Yeast *Saccharomyces cerevisia*, Virology 182 (1991) 513-521
132. Atlan, D., Portalier, R.: Purification of extracellular alkaline phosphatase released by *Escherichia coli* excretory mutants, Appl. Microbiol. Biotechnol. 26 (1987) 318-322
133. Fognini-Lefebvre, N., Portalier, R.: Synthèse et excrétion de β-lactamase chez une souche sauvage non excrétrice et un mutant excréteur d'*Escherichia coli* K-12, C.R. Acad. Sc. Paris t.296 (1983) 111-119
134. Mosbach, K., Birnbaum, S., Hardy, K., Davies, J., Bülow, L.: Formation of proinsulin by immobilized *Bacillus subtilis*, Nature 302 (1983) 543-545
135. Sampson, B.A., Benson, A.: *Escherichia coli* strains with defined mutations which alter cellular permeability, J. Ind. Microb. 1 (1987) 335-340
136. Nielsen, O.J., Costa-Giomi, P., Weinmann, R., Erslev, A.J., Caro, J.: Erytropoietin-β-D-Galactosidase - the Generation, Purification and Use of a Fusion Protein, J. Immun. Meth. 111 (1988) 1-9
137. Lin, Y., Robb, R.J., Gray, J.E., Simon, P.: The Construction and Characterization of a Biologically Active Recombinant IL-2 Containing a Lysine-Rich C-Terminal Extension, J. Immunol. 141 (11) (1988) 3847-3851
138. Lowe, C.R.: New developments in downstream processing, J. Biotechnol. 1 (1984) 3-12
139. Narayanan, S.R., Crane, L.J.: Affinity Chromatography Supports: A Look at Performance Requirements, Trends Biotechnol. 8 (1990) 12-16
140. Plückthun, A.: Antibody Engineering: Advances from the Use of *Escherichia coli* Expression Systems, Bio/Technology 9 (1991) 545-551
141. Vijayalakshmi, M.A., Bertrand, O. (Eds.): Protein-Dye Interactions, Developments and Applications, Elsevier Appl. Science, London - New York 1989
142. Nilsson, B., Abrahamsén, L.: Fusions to *Staphylococcal* Protein A, Methods Enzymol. 185 (1990) 144-161
143. Joice J.F.: Directed Molecular Evolution, Sci. Amer. 267(6) (1992) 48-55
144. Tuerk, C., Gold, L.: Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase, Science 249 (1990) 505-510
145. Porath, J., Olin, B.: Immobilized Metal Ion Affinity Adsorption and Immobilized Metal Ion Affinity Chromatography of Biomaterials. Serum Protein Affinities for Gel-Immobilized Iron and Nickel Ions, Biochem. 22 (1983) 1621-1630
146. Kågedal, L.: Immobilized Metal Ion Affinity Chromatography, in: [10] 227-251
147. Hochuli, E., Döbeli, H., Schacher, A.: New Metal Chelate Adsorbent Selective for Proteins and Peptides Containing Neighbouring Histidine Residues, J. Chromatogr. 411 (1987) 177-184

148. Bacolod, M.D., El Rassi, Z.: High-Performance Metal Chelate Interaction Chromatography of Proteins with Silica-Bound Ethylene Diamine-N,N'-Diacetic Acid, J. Chromatogr. 512 (1990) 237-247
149. Sulkowski, E.: Purification of Proteins by IMAC, Trends Biotechnol. 3 (1) (1985) 1-7
150. Hutchens, T.W., Yip, T.-T.: Anal. Biochem. 191 (1990) 160-168
151. Hochuli, E.: Large-Scale Chromatography of Recombinant Proteins, J. Chromatogr. 444 (1988) 293-302
152. Lillehoj, E.P., Malik, V.S.: Protein Purification, Adv. Biochem. Eng./Biotechnol. 40 (1989) 19-71
153. Chisti, Y., Moo-Young, M.: Large-Scale Protein Separations: Engineering Aspects of Chromatography, Biotech. Adv. 8 (1990) 699-708
154. Yang, B.-L., Goto, M., Goto, S.: Enzyme Purification by Affinity Chromatography Combined with Batchwise Adsorption and Columnwise Elution, J. Chem. Eng. Japan 22 (5) (1989) 532-537
155. Somers, W., van't Riet, K., Rozie, H., Rombouts, F.M., Visser, J.: Isolation and Purification of Endo-Polygalactoronase by Affinity Chromatography in a Fluidized Bed Reactor, Chem. Eng. J. 40 (1989) B7-B19
156. Nigam, S.C., Sakoda, A., Wang, H.Y.: Bioproduct Recovery from Unclaryfied Broth and Homogenates Using Immobilized Adsorbents, Biotech. Progr. 4 (3) (1988), 166-172
157. Wells, C.M., Lyddiatt, A., Patel, K.: Liquid Fluidized Bed Adsorption in Biochemical Recovery from Biological Suspensions, in : [11] 217-224
158. Roe, S.D.: Whole broth extraction of enzymes from fermentation broths using commercially available adsorbents, in : [11] 210-216
159. Molinari, R., Torres, J.L., Michaels, A.S., Kilpatrick, P.K., Carbonell, R.G.: Simultaneous Ultrafiltration and Affinity Sorbtive Separation of Proteins in a Hollow-Fibre Membrane Module, Biotechnol. Bioeng. 36 (1990) 572-580
160. Aigner, H., Brandt, S., Goffe, R., Kessler, S., O'Conner, J., Zale, S.: Proteinreinigung durch Affinitätsverfahren und Membranen, BioTec 2 (2) (1991) 66-69
161. Briefs, K.-G.: Proteinchromatographie mit modifizierten Membranen, Fortschritt-Berichte VDI, Reihe 17: Biotechnik, Nr. 72, VDI-Verlag, Düsseldorf 1991
162. Biosep. 1 (3-4) (1990) (Special Issue: Aqueous two-phase separation systems)
163. Bückmann, A.F., Morr, M., Kula, M.-R.: Preparation of Technical Grade Polyethylene Glycol (PEG) (MT_r 20000)-N-(2-Aminoethyl)-NADH by a Procedure Adaptable to Large-Scale Synthesis, Biotech. Appl. Biochem. 9 (1987) 258-268
164. Wuenschell, G.E., Naranjo, E., Arnold, F.H.: Aqueous Two-Phase Metal Affinity Extraction of Heme Proteins, Bioprocess Eng. 5 (1990) 199-202
165. Schustolla, D., Ledoux, C., Papamichael, N., Hustedt, H.: Reactive (Affinity) Extraction of Enzymes from Biomass, Ber. Bunsenges. Phys. Chem. 93 (1989) 971-975

166. Chen, J.-P.: Novel Affinity-Based Processes for Protein Purification, J. Ferment. Bioeng. 70 (3) (1990) 199-209
167. Johansson, G., Tjerneld, F.: Affinity partition between aqueous phases - a tool for large-scale purification of enzymes, J. Biotechnol. 11 (1989) 135-142
168. Schustolla, D., Hustedt, H.: Affinity extraction of proteins from biomass - metal affinity extraction of D-2-hydroxyisocaproate dehydrogenase from *Lactobacillus casei*, DECHEMA Biotech. Conf. 4B (1990) 1137-1141
169. Woll, J.M., Hatton, T.A., Yarmush, M.L.: Bioaffinity separations using reversed micellar extraction, Biotech. Progr. 5 (2) (1989) 57-62
170. van Dam, M.E., Wuenschell, G.E., Arnold, F.H.: Metal Affinity Precipitation of Proteins, Biotech. Appl. Biochem. 11 (1989) 492-502
171. Pécs, M., Eggert, M., Schügerl, K.: Affinity precipitation of extracellular microbial enzymes, J. Biotechnol. 21 (1991) 137-142
172. Flaschel, E., Wandrey, C., Kula, M.-R.: Ultrafiltration for the Separation of Biocatalysts, Adv. Biochem. Eng./Biotechnol. 26 (1983) 74-142
173. Ivory, C.F.: The Prospects for Large-Scale Electrophoreses, Sep. Sci. Technol. 23 (8-9) (1988) 875-912
174. Nath, S., Schütte, H., Hustedt, H., Deckwer, W.-D.: Continuous Separation of Proteins by Free-Flow Zone Electrophoresis, Ber. Bunsenges. Phys. Chem. 93 (1989) 1042-1046
175. Rutte, R.: Free Flow Electrophoresis as a Purification Step of High Versatility for Bioproducts, Ber. Bunsenges. Phys. Chem. 93 (1989) 1038-1042
176. Righetti, P.G.: Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 20, Elsevier, Amsterdam 1990
177. MEDAC: pAX, a universal DNA vector system for gene expression, sequencing, and mutagenesis of gene fusion proteins, Hamburg 1991
178. International Biotechnologies, Inc. (Kodak): The FLAG Biosystem, New Haven 1991
179. Moks, T., Abrahmsén, L., Österlöf, B., Josephson, S., Östling, M., Enfors, S.-O., Persson, I.-L., Nilsson, B., Uhlén, M.: Bio/Technology 5 (1987) 379-382
180. DIAGEN GmbH (QIAGEN, Inc.) : The QIAexpressionist, Düsseldorf 1991
(Other sources: Invitrogen, San Diego; Novagen, Madison)
181. Köhler, K., Veide, A., Enfors, S.-O.: Partitioning of β-Galactosidase Fusion Proteins in PEG/Potassium Phosphate Aqueous Two-Phase Systems, Enzyme Microb. Technol. 13 (1991) 204-209
182. Veide, A., Strandberg, L., Enfors, S.-O.: Extraction of β-galactosidase fused protein A in aqueous two-phase systems, Enzyme Microb. Technol. 9 (1987) 730-738
183. Köhler, K., Ljungquist, C., Kondo, A., Veide, A., Nilsson, B.: Engineering Proteins to Enhance their Partition Coefficients in Aqueous Two-Phase Systems, Bio/Technology 9 (1991) 642-646

184. Suh, S.-S., Haymore, B.L., Arnold, F.H.: Characterization of His-X_3-His sites in α-Helices of Synthetic Metal-Binding Bovine Somatotropin, Protein Eng. 4 (3) (1991) 301-305

185. Parker, D.E., Glatz, C.E., Ford, C.F., Gendel, S.M., Suominen, I., Rougvie, M.A.: Recovery of a Charged-Fusion Protein from Cell Extracts by Polyelectrolyte Precipitation, Biotechnol. Bioeng. 36 (1990) 467-475

186. Zhao, J., Ford, C.F., Glatz, C.E., Rougvie, M.A., Gendel, S.M.: Polyelectrolyte Precipitation of β-Galactosidase Fusions Containing Poly-Aspartic Acid Tails, J. Biotechnol. 14 (1990) 273-283

187. Suh, S.-S., Van Dam, M.E., Wuenschell, G.E., Plunkett, S., Arnold, F.H.: Novel Metal-Affinity Protein Separations, ACS Symp. Ser. 427 (1990) 139-149

188. Taylor, M.E., Drickamer, K.: Carbohydrate-Recognition Domains as Tools for Rapid Purification of Recombinant Eukaryotic Proteins, Biochem. J. 274 (1991) 575-580

189. Hopp, T.P., Prickett, K.S., Price, V.L., Libby, R.T., March, C.J., Cerretti, D.P., Urdal, D.L., Conlon, P.J.: A Short Polypeptide Marker Sequence Useful for Recombinant Protein Identification and Purification, Bio/Technology 6, (1988) 1204-1210

190. Hanada, K., Yamamoto, I., Anraku, Y.: Construction and Properties of Bifunctionally Active Membrane-Bound Fusion Proteins, J. Biol. Chem. 262 (1987) 14100-14104 , Purification and Reconstitution of *Escherichia coli* Proline Carrier Using a Site Specifically Cleavable Fusion Protein, *ibid* 263 (15) (1988) 7181-7185

191. Hickman, R.K., McMurry, L.M., Levy, S.B.: Overproduction and Purification of the Tn10-Specific Inner Membrane Tetracycline Resistance Protein Tet using Fusions to β-Galactosidase, Mol. Microbiol. 4 (7) (1990) 1241-1251

192. Nilsson, B., Abrahamsén, L., Uhlén, M.: Immobilization and Purification of Enzymes with Staphylococcal Protein A Gene Fusion Vectors, EMBO J. 4 (1985) 1075-1080

193. Nilsson, B., Holmgren, E., Josephson, S., Gatenbeck, S., Philipson, L., Uhlén, M.: Efficient Secretion and Purification of Human Insulin-Like Growth Factor I With a Gene Fusion Vector in *Staphylococci*, Nucl. Acids Res. 13 (4) (1985) 1151-1162

194. JP 03,080,086, 4. Apr. 1991, Chisso Corp. (Aoyama S., Erizumi A., Yoshino S., Inoue S., Yoshida N.)

195. Baneyx, F., Schmidt, C., Georgiou, G.: Affinity immobilization of a genetically engineered bifunctional hybrid protein, Enzyme Microb. Technol. 12 (1990) 337-342

196. Soehlemann, P., Oeckl, C., Michel, H.: Expression in *Escherichia coli* of the genes coding for reaction center subunits from *Rhodobacter sphaeroides*: wild-type proteins and fusion proteins containing one or four truncated domains from *Staphylococcus aureus* protein A at the carboxy-terminus, Biochim. Biophys. Acta 1089 (1) (1991) 103-112

197. Ullman, A.: One-step purification of hybrid proteins which have β-galactosidase activity, Gene 29 (1984) 27-31

198. Germino, J., Bastia, D.: Rapid Purification of a Cloned Gene Product by Genetic Fusion and Side-Specific Proteolysis, Proc. Natl. Acad. Sci. USA 81 (1984) 4692-4696

199. Scholtissek, S., Grosse, F.: A Plasmid Vector System for the Expression of a Triprotein Consisting of β-Galactosidase, a Collagenase Recognition Site and a Foreign Gene Product, Gene 62 (1988) 55-64

200. Brandsch, R., Bichler, V.: Autoflavinylation of Apo6-Hydroxy-D-Nicotin Oxydase, J. Biol. Chem. 266 (28) (1991) 19 056-19 062

201. WO 84 04,756, 06. Dec. 1984 (EP 0,131,363, 16. Jan. 1985) Celltech Ltd. (Bennett A.D., Rhind S.K., Lowe P.A., Hentschel C.C.G.: Polypeptide and Protein Products, and Processes for their Production and Use)

202. Dykes, C.W., Bookless, A.B., Coomber, B.A., Noble, S.A., Humber, D.C., Hobden, A.N.: Expression of Atrial Natriuratic Factor as a Cleavable Fusion Protein with Chloramphenicol Acetyltransferase in *Escherichia coli*, Eur. J. Biochem. 174 (1988) 411-416

203. Lew, A.M., Beck, D.J., Thomas, L.M.: Recombinant Fusion Proteins of Protein A and Protein G with Glutathione S-Transferase as Reporter Molecules, J. Immun. Meth. 136 (1991) 211-219

204. Smith, D.B., Johnson, K.S.: Single-Step Purification of Polypeptides Expressed in *Escherichia coli* as Fusions with Glutathione S-Transferase, Gene 67 (1988) 31-40

205. Chen, L., Ford, C., Kusnadi, A., Nikolov, Z.L.: Improved Adsorption to Starch of a β-Galactosidase Fusion Protein Containing the Starch-Binding Domain from *Aspergillus* Glucoamylase, Biotechnol. Progr. 7 (1991) 225-229

206. Ong, E., Gilkes, N.R., Warren, R.A.J., Miller, R.C., Kilburn, D.G.: Enzyme immobilization using the cellulose-binding domain of a *cellulomonas fimi* exoglucanase, Bio/Technology 7 (1989) 604-607

207. Ong, E., Gilkes, N.R., Miller, R.C., Warren, R.A.J., Kilburn, D.G.: Enzyme Immobilization Using a Cellulose-Binding Domain: Properties of a β-Glucosidase Fusion Protein, Enzyme Microb. Technol. 13 (1) (1991) 59-65

208. Ong, E., Greenwood, J.M., Gilkes, N.R., Kilburn, D.G., Miller, R.C., Warren, R.A.: The Cellulose-Binding Domains of Cellulases: Tool for Biotechnology, Trends Biotechnol. 7 (1989) 239-243

209. WO 86 02,077, 10. Apr. 1986 (US 656,873, 02. Okt. 1984) Biogen N.V. (Meade H.M., Garwin J.L.)

210. Nygren, P.Å., Eliasson, M., Abrahmsén, L., Uhlén, M., Palmcrantz, E.: Analysis and Use of the Serum Albumin Binding Domains of Streptococcal Protein G, J. Mol. Recognit. 1 (2) (1988) 69-74

211. Maina, C.V., Riggs, P.D., Grandea, A.G., Slatko, B.E., Moran, L.S., Tagliamonte, J.A., McReynolds, LA., di Guan, C.: An *Escherichia coli* Vector to Express and Purify Foreign Proteins by Fusion to and Separation from Maltose-Binding Protein, Gene 74 (1988) 365-373

212. Blondel, A., Bedouelle, H.: Engineering the Quaternary Structure of an Exported Protein with a Leucine Zipper, Protein Eng. 4 (4) (1991) 457-461
213. Abate, C., Luk, D., Gentz, R., Rauscher, F.J., Curran, T.: Expression and Purification of the Leucine Zipper and DNA-Binding Domains of Fos and Jun: Both Fos and Jun Contact DNA Directly, Proc. Natl. Acad. Sci. USA 87 (1990) 1032-1036
214. le Grice, F.J., Grüninger-Leitch, F.: Rapid Purification of Homodimer and Heterodimer HIV-1 Reverse Transcriptase by Metal Chelate Affinity Chromatography, Eur. J. Biochem. 187 (1990) 307-314
215. Smith, M.C., Furman, T.C., Ingolia, T.D., Pidgeon, C.: Chelating Peptide-Immobilized Metal Ion Affinity Chromatography, J. Biol. Chem. 263 (15) (1988) 7211-7215
216. Ljungquist, C., Breitholtz, A., Brink-Nilsson, H., Moks, T., Uhlén, M., Nilsson, B.: Immobilization and Affinity Purification of Recombinant Proteins Using Histidine Peptide Fusions, Eur. J. Biochem. 186 (1989) 563-569
217. Brewer, S.J., Sassenfeld, H.M.: The Purification of Recombinant Proteins Using C-Terminal Polyarginin Fusions, Trends Biotechnol. 3 (5) (1985) 119-122
218. Sassenfeld, H.M., Brewer, S.J.: A Polypeptide Fusion Designed for the Purification of Recombinant Proteins, Bio/Technology 2 (1984) 76-81
219. Persson, M., Bergstrand, M.G., Bülow, L., Mosbach, K.: Enzyme Purification by Genetically Attached Polycysteine and Polyphenylalanine Affinity Tails, Anal. Biochem. 172 (1988) 330-337
220. Carter, P., Wells, J.A.: Engineering Enzyme Specificity by "Substrate-Assisted Catalysis", Science 237 (1987) 394-399
221. Bouvet, P., Belasco, J.G.: Control of RNase E-mediated RNA degradation by 5'-terminal base pairing in E.coli, Nature 360 (1992) 488-491
222. Enfors, S.-O.: Control of *in vivo* proteolysis in the production of recombinant proteins, Trends Biotechnol. 10 (1992) 310-315
223. EP 0'300'425 A2, 25. 01. 1989, Boehringer Mannheim GmbH (Kopetzki E., Schumacher G.: Verfahren zur Herstellung von Proteinen in löslicher Form)
224. Hartmann, J., Daram, P., Frizzell, R.A., Rado, T., Benos, D.J., Sorscher, E.J.: Affinity Purification of Insoluble Recombinant Fusion Proteins Containing Glutathione-S-Transferase, Biotechnol. Bioeng. 39 (1992) 828-832
225. Radford, S.E., Dobson, C.M., Evans, P.A.: The folding of hen lysozyme involves partially structured intermediates and multiple pathways, Nature 358 (1992) 302-307
226. Ellis, J.: Cytosolic chaperonin confirmed, Nature 358 (1992) 191-192
227. Yaffe, M.B., Farr, G.W., Miklos, D., Horwich, A.L., Sternlicht, M.L., Sternlicht, H.: TCP1 complex is a molecular chaperone in tubulin biogenesis, Nature 358 (1992) 245-248
228. Lewis, V.A., Hynes, G.M., Zheng, S., Saibil, H., Willison, K.: T-complex polypeptide-1 is a subunit of a heteromeric particle in the eukaryotic cytosol, Nature 358 (1992) 249-252

229. Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M.K., Hartl, F.U.: Successive action of DnaK, DnaJ and GroEL along the pathway of chaperone-mediated protein folding, Nature 356 (1992) 683-689

230. Lee, S.C., Olins, P.O.: Effect of Overproduction of Heat Shock Chaperones GroESL and DnaK on Human Procollagenase Production in *Escherichia coli*, J. Biol. Chem. 267 (5) (1992) 2849-2852

231. Sherman, M.Y., Goldberg, A.L.: Heat shock in *Escherichia coli* alters the protein-binding properties of the chaperonin groEL by inducing its phosphorylation, Nature 357 (1992) 167-169

232. Buchner, J., Brinkmann, U., Pastan, I.: Renaturation of a single-chain immunotoxin facilitated by chaperones and protein disulfide isomerase, Bio/Technol. 10 (1992) 682-685

233. Schönbrunner, E.R., Schmid, F.X.: Peptidyl-prolyl cis-trans isomerase improves the efficiency of protein disulfide isomerase as a catalyst of protein folding, Proc. Natl. Acad. Sci. USA 89 (1992) 4510-4513

234. Kamitani, S., Akiyama, Y., Ito, K.: Identification and characterization of an *Escherichia coli* gene required for the formation of correctly folded alkaline phosphatase, a periplasmic enzyme, EMBO J. 11 (1) (1992) 57-62

235. Forsberg, G., Baastrup, B., Rondahl, H., Holmgren, E., Pohl, G., Hartmantis, M., Lake, M.: An Evaluation of Different Enzymatic Cleavage Methods for Recombinant Fusion Proteins, Applied on Des(1-3)Insulin-Like Growth Factor I, J. Prot. Chem. 11 (2) (1992) 201-211

236. Ishizako, J., Tamaki, M., Shin, M., Tsuzuki, H., Yoshikawa, K., Teraoka, H., Yoshida, N.: Production of recombinant human glucagon in the form of a fusion protein in *Escherichia coli*; recovery of glucagon by sequence-specific digestion, Appl. Microbiol. Biotechnol. 36 (1992) 483-486

237. Luirink, J., High, S., Wood, H., Giner, A., Tollervey, D., Dobberstein, B.: Signal-sequence recognition by an *Escherichia coli* ribonucleoprotein complex, Nature 359 (1992) 741-743

238. Phillips, G.J., Silhavy, T.J.: The *E. coli ffh* gene is necessary for viability and efficient protein export, Nature 359 (1992) 744-746

239. Harwood, C.R.: *Bacillus subtilis* and its relatives: molecular biological and industrial workhorses, Trends Biotechnol. 10 (1992) 247-256

240. Reiser, J., Glumoff, V., Kälin, M., Ochsner, U.: Transfer and Expression of Heterologous Genes in Yeasts Other Than *Saccharomyces cerevisiae,* Adv. Biochem. Eng./Biotechnol. 43 (1990) 75-102

241. Gellissen, G., Strasser, A.W.M., Melber, K., Merckelbach, A., Weydemann, U., Keup, P., Dahlems, U., Piontek, M., Hollenberg, C.P., Janowicz, Z.A.: Die methylotrophe Hefe *Hansenula polymorpha* als Expressionssystem für heterologe Proteine, BioEng. 5(1990) 20-26

242. van den Berg, J.A., van der Laken, K.J., van Ooyen, A.J.J., Renniers, T.C.H.M., Rietveld, J., Schaap, A., Brake, A.J., Bishop, R.J., Schultz, K., Moyer, D., Richmann, M., Shuster, J.R.: *Kluyveromyces* as a host for heterologous gene expression: expression and secretion of prochymosin, Bio/Technol. 8 (1990) 135-139

243. Heslot, H.: Genetics and Genetic Engineering of the Industrial Yeast *Yarrowia lipolytica*, Adv. Biochem. Eng./Biotechnol. 43 (1990) 43-73

244. Nesmeyanova, M.A., Karmamyshev, A.L., Tsfasman, I.M., Fedotikova, E.T.: Transformation of different strains of *Escherichia coli* with multicopy plasmids carrying the phoA gene causes the secretion of periplasmic alkaline phosphatase and the accumulation of its precursor, Mol. Biol. 25 (3) (1991) 624-631

245. Edgington, S.M.: Rites of Passage: Moving Biotech Proteins Through The ER, Bio/Technol. 10 (1992) 1413-1420

246. Hanke, C., Hess, J., Schumacher, G., Goebel, W.: Processing by OmpT of fusion proteins carrying the HlyA transport signal during secretion by the *Escherichia coli* hemolysin transport system, Mol. Gen. Genet. 233 (1992) 42-48

247. Nakayama, A., Shimada, H., Furutani, Y., Honjo, M.: Processing of the prepropeptide portions of the *Bacillus amyloliquefaciens* neutral protease fused to *Bacillus subtilis* α-amylase and human growth hormone during secretion in *Bacillus subtilis*, J. Biotechnol. 23 (1992) 55-70

248. Tokunaga, M., Kawamura, A., Omori, A., Hishinuma, F.: Structure of yeast pGKL 128-kDa killer-toxin secretion signal sequence, Eur. J. Biochem. 203 (1992) 415-423

249. Wels, W., Harwerth, I.-M., Zwickl, M., Hardman, N., Groner, B., Hynes, N.E.: Construction, bacterial expression and characterization of a bifunctional single-chain antibody-phosphatase fusion protein targeted to the human erbB-2 receptor, Bio/Technology 10 (1992) 1128-1132

250. WO 92/01707 (06. 02. 1992), Bionebraska, Inc. (Coolidge T.R., Wagner F., van Heeke G., Schuster S.M., Stout J., Wylie D.: Purification directed cloning of peptides)

251. Su, X., Prestwood, A.K., McGraw, R.A.: Production of Recombinant Porcine Tumor Necrosis Factor Alpha in a Novel *E. coli* Expression System, Bio/Technol. 13 (5) (1992) 756-761

252. Engel, H., Mottl, H., Keck, W.: A Modified Vector for the Controlled High-Level Overproduction of Staphylococcal Protein A Fusion Proteins in the Periplasm of *Escherichia coli*, Protein Expression Purification 3 (1992) 108-113

253. Rondahl, H., Nilsson, B., Holmgren, E.: Fusions to the 5' end of a gene encoding a two-domain analogue of staphylococcal protein A, J. Biotechnol. 25 (1992) 269-287

254. Smith, M.C.: Engineering Metal Binding Sites into Recombinant Proteins for Facile Purification, Annals New York Acad. Sci. 646 (1991) 315-321

255. WO 92/07868 (14. 05. 1992), Pharmacia AB (Enfors S.-O., Köhler K., Ljungquist C., Nilsson B.: A method for isolating and purifying peptides and proteins)

256. Ortega, S., García, J.L., Zazo, M., Varela, J., Munoz-Willery, I., Cuevas, P., Giménez-Gallego, G.: Single-Step Purification on DEAE-Sephacel of Recombinant Polypeptides Produced in *Escherichia coli*, Bio/Technol. 10 (1992) 795-798

257. Sanchez-Puelles, J.M., Sanz, J.M., Garcia, J.L., Garcia, E.: Immobilization and single-step purification of fusion proteins using DEAE-cellulose, Eur. J. Biochem. 203 (1992) 153-159

258. Wolber, V., Maeda, K., Schumann, R., Brandmeier, B., Wiesmüller, L., Wittinghofer, A.: A universal expression-purification system based on the coiled-coil interaction of myosin heavy chain, Bio/Technol. 10 (1992) 900-904

259. Van Dyke, M.W., Sirito, M., Sawadogo, M.: Single-step purification of bacterially expressed polypeptides containing an oligo-histidine domain, Gene 111 (1992) 99-104

260. WO 91/15589 (17. 10. 1991), Upjohn Co. (Sharma S.K.: Improved Process of Purifying Recombinant Proteins and Compounds Useful in Such Process)

261. Clement, J.M., Charbit, A., Leclerc, C., Martineau, P., Muir, S., O'Gallaghan, D.; Molecular and cellular targeting in the expression of foreign polypeptides in bacteria, Antonie Leeuwenhoek J. Microbiol. 61 (1992) 143-152

262. Duvoisin, R.M., Belin, D., Krisch, M.: A plasmid expression vector that permits stabilization of both mRNAs and proteins encoded by the cloned genes, Gene 45 (1986) 193-201

263. Freudl, R.: Protein secretion in Gram-positive bacteria, J. Biotechnol. 23 (1992) 231-240

264. Georgellis, D., Arvidson, S., von Gabain, A.: Decay of ompA mRNA and processing of 9S RNA are immediately affected by shifts in growth rate, but in opposite manners, J. Bacteriol. 174 (1992) 5382-5390

265. Glisin, V., Francetic, O.: Enhanced production and recovery of recombinant proteins genetically targeted into the periplasm - *E. coli* penicillin acylase as an initial model system, Biol. Recomb. Microorg. Anim. Cells - Oholo 34th Meeting (1991) 71-76

266. Hernan, R.A., Hui, H.L., Andracki, M.E., Noble, R.W., Sligar, S.G., Walder, R.Y.: Human hemoglobin expression in *Escherichia coli*: importance of optimal codon usage, Biochem. 31 (1992) 8619-8628

267. Hlodan, R., Craig, S., Pain, R.H.: Protein folding and its implications for the production of recombinant proteins, Biotechnol. Genet. Eng. Rev. 9 (1991) 47-88

268. JP 4179-495: 14. 11. 90 & JP 309'912, 26. 06. 1992, MD-Research (Fusion protein cleavage for new fusion protein)

269. Keil, B.: Specifity of proteolysis, Springer Verlag, Berlin 1992

270. Kiefhaber, T., Rudolph, R., Kohler, H.-H., Buchner, J.: Protein aggregation *in vitro* and *in vivo*: a quantitative model of the kinetic competition between folding and aggregation, Bio/Technol. 9 (1991) 825-829

271. Kosinski, M.J., Rinas, U., Bailey, J.E.: Proteolytic response to the expression of an abnormal β-galalactosidase in *Escherichia coli*, Appl. Microbiol. Biotechnol. 37 (1992) 335-341

272. Murby, M., Cedergren, L., Nilsson, J., Nygren, P.-A., Hammarberg, B., Nilsson, B., Enfors, S.-O., Uhlén, M.: Stabilization of recombinant proteins from proteolytic degradation in *Escherichia coli* using a dual affinity fusion strategy, Biotechnol. Appl. Biochem. 14 (1991) 336-346

273. Nilsson, B., Berman-Marks, C., Kuntz, I.D., Anderson, S.: Secretion incompetence of bovine pancreatic trypsin inhibitor expressed in *Escherichia coli*, J. Biol. Chem. 266 (1991) 2970-2977

274. Orr, T.: Cell-free translation systems may provide new bioprocessing techniques, Gen. Eng. News (March 1989) 13 & 48

275. Pohlner, J., Klauser, T., Kuttler, E., Halter, R.: Sequence-specific cleavage of protein fusion using recombinant *Neisseria* type 2 IgA protease, Bio/Technol. 10 (1992) 799-804

276. Power, B.E., Ivancic N., Harley, V.R., Webster, R.G., Kortt, A.A., Hudson, P.J.: High-level temperature-induced synthesis of an antibody VH-domain in *Escherichia coli* using the PelB secretion signal, Gene 113 (1992) 95-99

277. Rinas, U., Tsai, L.B., Lyons, D., Fox, G.M., Stearns, G., Fieschko, J., Fenton, D., Bailey, J.E.: Cysteine to serine substitutions in basic fibroblast growth factor: effect on inclusion body formation and proteolytic susceptibility during *in vitro* refolding, Bio/Technol. 10 (1992) 435-440

278. Seeboth, P.G., Warren, R.A.J., Heim, J.: In vitro cleavage of a fusion protein bound to cellulose using soluble yscFs (Kex2) variant, Appl. Microbiol. Biotechnol. 37 (1992) 621-625

279. Tolentino, G.J., Meng, S.-Y., Bennett, G.N., San, K.-Y.: A pH regulated promoter for the expression of recombinant proteins in *Escherichia coli*, Biotechnol. Lett. 14 (1992) 157-162

280. Ward, E.S.: Antibody engineering: the use of *Escherichia coli* as an expression host, FASEB J., 6 (1992) 2422-2427

281. Studier, F.W., Moffatt, A.: Use of Bacteriophage T7 RNA Polymerase to Direct Selective High-Level Expression of Cloned Genes, J. Mol. Biol. 189 (1986) 113-130

282. Hellman, J., Mäntsälä, P.: Construction of an *Escherichia coli* export-affinity vector for expression and purification of foreign proteins by fusion to cyclomaltodextrin glucanotransferase, J. Biotechnol. 23 (1992) 19-34

283. Yu, P., San, K.Y.: Protein Release in Recombinant *Escherichia coli* Using Bacteriocin Release Protein, Biotechnol. Prog. 8 (1992) 25-29