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REVIEW

Upstream Strategies to Minimize Proteolytic Degradation upon Recombinant Production in *Escherichia coli*

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Proteolytic degradation of recombinant proteins represents a major problem related to production of gene products in heterologous hosts. Recombinant DNA technology offers several alternative strategies for stabilization of expressed gene products. These strategies can often give dramatic stabilization effects and can be combined with strategies involving optimization of fermentation conditions or downstream processing schemes. In this review, various genetic approaches to improve the stability of recombinant proteins will be discussed, including (i) choice of host cell strain, (ii) product localization, (iii) use of gene fusion partners, and (iv) product engineering. In addition, the solubility of the gene product can be influenced by factors such as growth temperature, promoter strength, fusion partners, and site-directed changes. Altogether, a battery of approaches can be used to obtain stable gene products.

When producing mammalian proteins in a bacterial host such as *Escherichia coli*, proteolytic degradation of the gene product might lead to significantly reduced yields and difficulties in setting up cost-effective production schemes. The degradation might occur during the cell harvest and recovery procedures but is normally initiated during the cultivation. A number of different strategies to decrease the proteolytic degradation of the product by modification of the downstream processing schemes or by optimization of fermentation conditions have earlier been presented (1–4). However, these strategies have in general rather limited influence on the quality of the product. In contrast, recombinant DNA techniques offer more directed strategies to minimize proteolysis. Both the host cell used for the production and the product can by genetic means be engineered to avoid proteolytic degradation. This review focuses mainly on these "upstream design" strategies, which can be either highly product specific or rather general in their nature.

The Mind Map for Recombinant E. coli Expression

A major consideration when setting up a production scheme for a recombinant protein is whether the product should be expressed intracellularly or if a secretion system could be used to direct the protein out of the host cell. There exists no generally applicable strategy that guarantees efficient expression. Instead, the inherent properties of the target protein decide which production route will be most successful, both in terms of expression level and in terms of product stability. The different production routes, important factors to consider, problems that can be encountered, and how these problems might be circumvented are presented in Fig. 1.

Fusion to a leader (signal) peptide can for certain gene products be used to direct the proteins out of the host cells (5,6). For *E. coli*, several systems have been described for the secretion of the gene product to the culture medium (7–12) or the periplasm (13–16). There are a number of advantages connected with the secretion strategy, including enhanced disulfide bond formation in the oxidative environment outside the cytoplasm, which might improve folding (9,15,17) and very much simplified recovery of the recombinant protein since a large degree of the purification has been achieved through the secretion (17,18). However, there is no guarantee that a certain protein can be secreted even if an expression system with secretion signals is chosen for the production. Proteins with a strong tendency to precipitate intracellularly and proteins containing hydrophobic transmembrane regions have been

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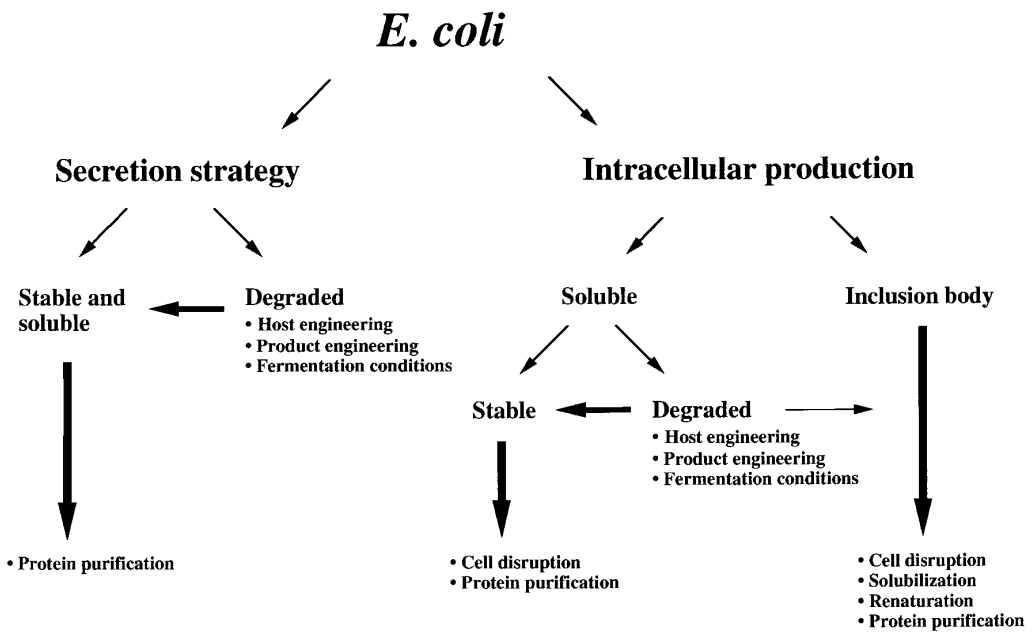

FIG. 1. The mind map for recombinant *E. coli* production. Factors like product solubility and stability together with possibilities for secretion decide which route will be the most suitable for production of the target protein. As can be seen at the bottom, the number of essential unit operations will depend upon the selected production route.

demonstrated to be difficult to secrete (19,20). Intracellular expression of nonsecretable proteins might be an attractive alternative due to recent advances for *in vitro* renaturation of recombinant proteins from intracellular precipitates (21).

If a secretion strategy proves to be successful, meaning that the product can be secreted out to the culture medium and at the same time no proteolytic degradation occurs, there exists no real obstacle to setting up a cost-efficient production scheme for the product. After a cell separation step, the gene product has to be separated from only a few other proteins and the culture medium constituents. If the product is partly degraded, different strategies exist to improve product stability (Fig. 1). If the product is not possible to secrete, an intracellular production scheme needs to be investigated. If a majority of the product is found in a soluble form and not degraded by host proteases, a relatively straight-forward recovery scheme could be envisioned, obviously initiated by a cell disruption step. If the soluble fraction of the intracellularly expressed protein is found to be degraded, again different strategies to stabilize the product can be evaluated (Fig. 1). If an intracellularly expressed gene product has a strong tendency to precipitate, proteolytic degradation is generally rather limited but the recovery scheme needs to include renaturation of the recombinant protein to yield a correctly folded product (21).

STABILIZATION STRATEGIES

An overview of some of the investigated strategies that have been employed to avoid proteolysis is presented in Table 1. A selection of examples involving strain and product engineering will be discussed below.

Choice of Host Cell

A strategy to enhance the *in vivo* stability of a labile recombinant protein is to use an *E. coli* host that lacks a protease or proteases, or other regulatory proteins, involved in the degradation (Table 1). There are several examples of this method in the literature (23–29,43–45). By using a *lon* strain, deficient in the La protease which is a cytoplasmic protease that seems to be largely responsible for degradation of proteins with nonnative structures (46), stabilization of the recombinant protein can be obtained (45). Strains that do not produce the alternate σ-factor, σ^{32}, which controls the transcription of heat shock genes, have been demonstrated to have a decreased capacity to degrade unstable or abnormal proteins, in addition to being defective in the heat shock response (28,45). It has also been shown that several chaperone proteins are involved in the protein degradation process, and the use of *dnaK*, *dnaJ*, *grpE*, or *groEL* mutant strains have been discussed as potential alternatives for expression of foreign, unstable proteins in *E. coli* (1,45,47). However, results from such studies do not suggest any general solutions, since although a *dnaK* deletion can have a stabilizing effect for one product (29), overexpression of the same chaperone can increase the *in vivo* half life 10-fold for another product (31). Determinations of how certain chaperones influence the stability of a given protein will have to be made empirically. Nevertheless, overproduction

TABLE 1
Upstream Strategies for Stabilization against Proteolysis

Strategy	Modification	Stabilized products	Refs.
A. Host cell engineering			
1. Protease deficient strains	DegP$^-$	Various fusion proteins: tsr-alkaline phosphatase, protein A–IL2, protein A–β-lactamase, ZZ-Ag332, etc.	22, 23, 24, 25
	OmpT$^-$	Protein A–β-galactosidase	26
	DegP$^-$, OmpT$^-$, Protease III$^-$	Protein A–β-lactamase	27
2. Heat-shock deficient strains	σ^{32-}	Fragments from *P. berghei* and *P. falciparum* antigens	28
	DnaK$^-$	Nonsecreted form of alkaline phosphatase	29
3. Chaperone coexpression	GroEL/ES expression	Mutant forms of the tetracycline/H$^+$ antiporter protein	30
	GroESL and DnaK expression	Human procollagenase	31
4. Protease inhibitor coexpression	T4 phage expression	β-Galactosidase	32
B. Product engineering			
1. Secretion	Signal sequence fusion	Proinsulin, human epidermal growth factor	33, 34
2. Inclusion body formation	Intracellular overexpression	RSV F fusion proteins, interferon-γ, and interleukin-2	19, 35
3. Single fusions	N- or C-terminal extensions	Human proinsulin, P22 Arc repressor, λ-repressor, human DNA excision-repair gene product	36, 37, 38, 39
4. Double fusions	N- and C-terminal extensions	IGF-II, proinsulin, rat protein disulfide isomerase, Vα region of a human T-cell receptor	37, 40
5. Protease site engineering	Site-directed mutagenesis	Protein A–β-galactosidase	26
6. Solubility engineering	Site-directed mutagenesis	Basic fibroblast growth factor, RSV G fusion proteins	41, 42

of the chaperones GroEL/ES has shown to increase the *in vivo* half life for certain products (30).

Since secretion systems are attractive for large-scale production of recombinant proteins, significant efforts have been devoted to the development of strains lacking proteases in the *E. coli* cell envelope (Table 1). Strauch and Beckwith (22) reported the use of a mutant *E. coli* strain lacking the DegP proteolytic activity in the periplasm, which gave a complete stabilization of a fusion protein comprising alkaline phosphatase and the inner membrane protein Tsr. There are now several reports showing that other recombinant proteins expressed in *E. coli* and secreted to the periplasm can also be stabilized when produced in a *degP*$^-$ strain (23–25).

A fusion between staphylococcal protein A (SpA) and *E. coli* β-galactosidase was demonstrated to be proteolytically processed when produced intracellularly in *E. coli* (26). A cleavage occurred in the linker region between the two functional units and the cleavage site, identified by N-terminal peptide sequencing, was found to be at two basic amino acids, Arg–Lys. The outer membrane protein T (OmpT), which recognizes and cleaves at dibasic amino acid sequences (48), was suggested to be responsible for this degradation since the degradation occurred during disintegration of the cells and the proteolytic activity was mapped to the outer membrane. When using an *ompT*$^-$ strain for the production, complete stabilization was indeed obtained (26). Similarly, an IGF-II fusion designed for secretion was degraded at basic amino acid sequences when produced in *E. coli* (40). An *ompT*$^-$ strain was also tried as production host for different IGF-II fusions, leading to significantly more stable products (49,50).

Along this line of research, *E. coli* strains with double and triple mutants of envelope proteases (27) have been created to be suitable production strains for secreted products. Unfortunately, by creating hosts deficient in multiple proteases, the viability and thus growth properties of the strain is impaired. Recently, a set of 20 different mutant strains was presented (44) in an attempt to generate *E. coli* host strains which exhibit the optimal combinations of growth properties and protein stability. If one has access to a set of such strains when setting up a production scheme for a recombinant product it is of course possible to test a number of strains in parallel to select a suitable host strain.

Secretion of the Gene Product

Gene fusions are widely used tools in biotechnology. By means of molecular biology, it has become possible to give proteins additional properties through gene fusions. A type of gene fusion which has been used extensively is the fusion of a signal sequence to the gene encoding the protein of interest, in order to localize the recombinant gene product to the periplasm and/or growth medium, with the primary purpose of simpli-

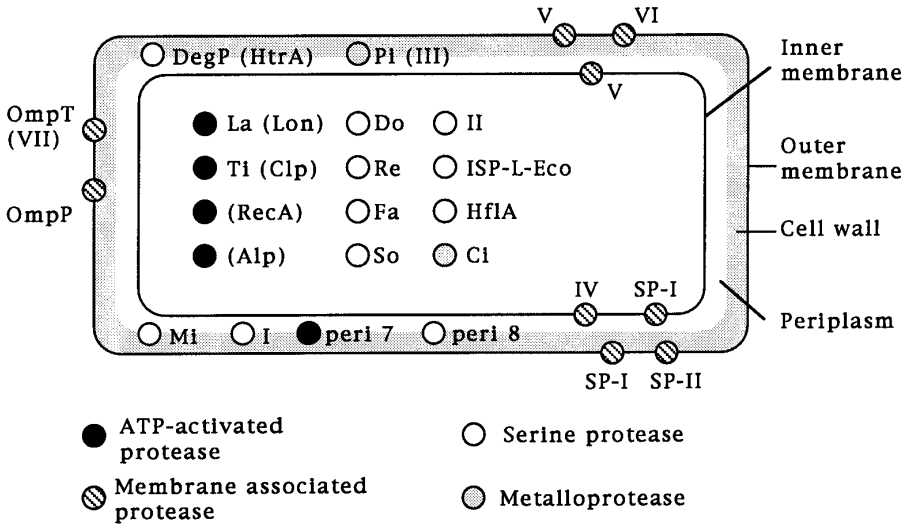

FIG. 2. Schematic distribution of *E. coli* proteases in the cell. The type of protease is indicated. The information is collected from recent reviews by Maurizi (51) and Goldberg (52).

fying product recovery. As mentioned above, there exist for *E. coli* a number of secretion systems that have proved beneficial, and the most attractive ones are of course those that secrete the protein to the culture medium (5,18).

More than 20 proteases have to date been identified in *E. coli* (Fig. 2), of which approximately 50% are localized in the cytoplasm. Extensive reviews have recently been published (51–53). By secretion to the periplasm or to the culture medium, stabilization of recombinant proteins can thus potentially be achieved. Talmadge and Gilbert (33) demonstrated that the *in vivo* half life of rat proinsulin increased 10 times when secreted to the periplasm, as compared to when located in the cytoplasm. However, this is not a general phenomenon and the direction of a recombinant protein to the secretion apparatus could in some cases lead to degradation (43,54). Obviously, the inherent properties of the recombinant protein determine whether the protein is stabilized by secretion or not. For example, stabilization due to translocation has been reported for fragments of the F glycoprotein from human respiratory syncytial virus (19), human epidermal growth factor (34), and human cysteine proteinase inhibitor (55). However, many recombinant proteins cannot be translocated through the *E. coli* inner membrane. They are either secretion incompetent or form inclusion bodies in the cytoplasm. When this is the case, an intracellular production strategy has to be used.

Improved Recovery via Inclusion Body Formation

The mechanisms for inclusion body formation are not fully elucidated but fusions to certain proteins, such as TrpE and to a lesser extent β-galactosidase, may result in inclusion body formation (56). In addition, expression from strong promoters such as the T7 promoter (57), leading to very high expression levels, seems to increase the tendency for intracellular aggregation of the produced recombinant protein (21,58). Production by the inclusion body strategy has the main advantages that the recombinant product normally is protected from proteolysis (21,59) and that it can be produced in large quantities. Levels of up to 50% of total cell protein content have been reported (21,26). If an intracellularly produced recombinant product exists as partly soluble and partly precipitated material, and the soluble material is significantly degraded, it is obviously of interest to increase the amount of precipitated material. This could be achieved by increasing the expression level by the use of strong promoter systems (e.g., the T7 system) and high copy number plasmids. By altering growth conditions that lead to increased gene expression, such as elevated growth temperature or gene induction level (60), an increase in inclusion body formation can also be obtained. Intracellular production and recovery from inclusion bodies have become more attractive due to recent advances for *in vitro* renaturation of recombinant proteins from intracellular precipitates (21) and gene fusion technology which has also contributed to strategies for recovery of proteins with low solubility (19).

In contrast, if no problems with proteolysis exist during intracellular production, it might be desired to obtain the recombinant protein in a soluble form (Fig. 1). Increased solubility might be achieved by lowering the growth temperature (2,3,60,61), by fusion to soluble fusion partner like the ZZ-tail from protein A (61,62) or thioredoxin (63) or by hydrophobicity engineering (41,42).

Gene Fusions for Product Stabilization

Fusions to homologous or heterologous genes have proved to provide stabilization of recombinant proteins. Unfused proinsulin showed a very short *in vivo* half life in the *E. coli* cytoplasm (33). In contrast, when the proinsulin gene was fused to a single copy of itself, a significant stabilization was achieved (36). Proinsulin has also been produced in a fairly stable form as a fusion to the IgG-binding ZZ-tail, although several degradation products were obtained together with the full length fusion protein after affinity chromatography (37). Bowie and Sauer (38) demonstrated that a mutant form of the phage repressor protein P22 Arc, expressed intracellularly, could be stabilized *in vivo* by a 25 amino acid C-terminal extension. This peptide also stabilized a labile mutant form of the λ-repressor when fused at the C-terminal. A eukaryotic protein, ubiquitin, has been used as a fusion partner for *E. coli* production of recombinant proteins. In contrast to its function in eukaryotic systems where it targets certain proteins to degradation, ubiquitin stabilizes the recombinant proteins in *E. coli* (39,65).

Fusion strategies can increase the stability of recombinant proteins produced in *E. coli* compared to the native protein, but nevertheless, heterogeneous mixtures of product molecules are often obtained after purification. In an attempt to avoid this problem a dual affinity fusion strategy was developed to enable specific recovery of only full length products (40). This strategy, where proteins of interest were fused between two affinity tags, the IgG-binding ZZ and the albumin binding ABP, resulted in a considerable stabilization of several mammalian proteins (IGF-II, proinsulin, a thioredoxin homologous domain of rat protein disulfide isomerase and a part (Vα) of a human T-cell receptor) which were all unstable when produced as single fusions (37,40). In addition, consecutive affinity purifications on IgG-Sepharose (using the ZZ fusion partner) and human serum albumin Sepharose (using the ABP fusion partner) yielded highly purified full length fusion proteins (37,40). Plasmid systems for *E. coli* expression of dual affinity fusions have been designed for both secreted and intracellular production (19,40). A related dual affinity system was developed for purification of staphylococcal protein A deletion mutants (66). The N- and C-terminal affinity handles were in this case the albumin-binding ABP and a Zn^{2+}-binding histidine tag, respectively. Also this strategy exhibited a stabilizing effect on the labile target protein, in addition to providing means for specific purification of full length proteins (66).

Protein Engineering to Avoid Proteolysis

When a proteolytically sensitive site is located in a region of a protein not important for activity, folding, or other functional properties, it might be possible to either delete or substitute amino acids in the recognition sequence to obtain a stable gene product. A fusion protein SpA–β-galactosidase was shown to be proteolytically digested by the OmpT-protease at a basic dipeptide sequence in the linker region between the two functional units (26). A modified SpA–β-galactosidase fusion lacking the basic amino acids retained IgG-binding and β-galactosidase activities. Stability studies on the modified fusion protein revealed that it was completely stable when incubated with *E. coli* cell disintegrate, as compared to the original fusion that was almost completely degraded after 4 h incubation under the same conditions (26). Similar strategies have also been employed in attempts to increase *in vitro* protein stability of therapeutical products such as insulin. Single amino acid substitutions have resulted in significantly improved product stability (61,67).

As discussed earlier, heterologous protein expression in recombinant hosts like *E. coli* often leads to accumulation of the protein in an improperly folded, nonactive form as inclusion bodies (21,68). This phenomenon can sometimes be advantageous due to increased protein stability (58,59). However, refolding schemes to obtain correctly folded proteins usually involve the use of strong denaturants like guanidine hydrochloride and urea to resolubilize the inclusion bodies, which can lead to unwanted chemical modifications of the recombinant protein. Guanidine hydrochloride is rather expensive and highly corrosive, which makes it less suitable for industrial use. The refolding is usually performed at low protein concentrations to avoid aggregation, generating large working volumes. Taken together, these properties can lead to high costs and extended processing schemes for the production and the recovery of recombinant proteins. Therefore, a major challenge in recombinant expression is to design schemes for soluble production. Four major routes to increase the solubility of recombinant proteins have so far been investigated. Lowering the growth temperature often yields a larger amount of soluble protein (60,61,69). It has also been suggested that coexpression of chaperones should lead to a larger fraction of soluble recombinant protein (70). This hypothesis is supported by some reports about coexpression of GroEL and GroES for production of active proteins in *E. coli* (30,71). A third alternative strategy is to fuse the protein of interest to a soluble fusion partner, leading to an increased solubility. Samuelsson and co-workers (62) have described the use of the SpA derivative ZZ to increase the solubility of human IGF-I. When IGF-I was fused to ZZ, the *in vitro* solubility was increased at least 100 times as compared to unfused IGF-I. The ZZ-tail has also been shown to be a useful tool in *in vitro* refolding of IGF-I, probably acting in a "chaperone-like" manner,

preventing aggregation of unfolded fusion proteins thus giving significantly reduced working volumes and more cost-effective downstream processing (63). LaVallie and co-workers (64) have used the highly soluble protein thioredoxin to produce large amounts of soluble recombinant proteins in the *E. coli* cytosol. A fourth strategy is to perform site-directed mutagenesis of specific residues to alter stability and solubility properties of recombinant proteins. Few systematic studies of this strategy are reported although such small specific modifications have been shown to alter properties like solubility and stability of the recombinant protein (26,41,60,61,68,72). Rinas and co-workers (41) showed that different single cysteine substitution in human basic fibroblast growth factor (bFGF) could cause either an increase or a decrease in solubility. Luck and co-workers (73) demonstrated that by replacing cysteines with serines in bovine prolactin, a significant increase in the protein solubility was achieved. Different strategies to alter protein solubility were recently reviewed (6).

Recently, site-directed mutagenesis was employed to engineer the hydrophobic properties of a 101 amino acid fragment from the human respiratory syncytial virus major glycoprotein (42). Hydrophobic engineering of four clustered phenylalanine residues, yielding mutant variants with the four phenylalanine residues either substituted (for serine residues) or deleted, increased the fraction of soluble protein *in vivo* from 27 to 75%. Interestingly, this effect was accompanied by a remarkable increase in product stability. Protein engineering of hydrophobic residues obviously could influence protein structure but in the reported example, circular dichroism analysis and antigenicity pattern suggested a retained structure (42). Similarly, the solubilizing effect observed for a Ser88Cys mutant of bFGF was accompanied by increased proteolytic stabilization (41). These results indicate that in certain cases, single amino acid substitutions and engineering of hydrophobic residues (by substitution for more hydrophilic residues) might be used as tools to increase the solubility and proteolytic stability of poorly soluble and labile proteins.

CONCLUSIONS

This review presents different strategies to use recombinant DNA technology for the stabilization of recombinant proteins expressed in *E. coli.* The use of a protease deficient production host is one attractive route to minimize proteolysis. Preferably, the protease responsible for the majority of the degradation should be mapped, and a strain lacking the protease could be tested. Double or triple protease mutant strains have not been used extensively due to impaired growth properties of many of these strains (27,44). If intracellular production is utilized, the gene product could be recovered either from inclusion bodies or from the fraction of soluble proteins, depending on inherent properties of the gene product and the expression rate. If a majority of the protein is found as inclusion bodies, the target protein is most probably protected from proteolytic degradation, but a renaturation scheme has to be set up for the recovery of correctly folded product. If a soluble protein is degraded in the cytoplasm, different strategies employing gene fusions could be investigated to achieve stabilization. A secretion strategy could be evaluated by fusing the target gene to signals resulting in secretion of the gene fusion product. Alternatively, single or dual fusions could be tested for product stabilization. Such fusion tags could be chosen also to enable affinity purification which would be beneficial for efficient product recovery. However, depending on the final use of the product, the fusion tails might have to be removed by chemical or enzymatic cleavage of the purified gene product (74–76). If proteolytic degradation at a specific site is predominant and the site can be defined, site-directed mutagenesis could be employed to create a slightly modified product that should be protected from proteolysis. Gene fusion technology or site-directed mutagenesis could also be utilized to alter the hydrophobic properties of the target protein, since in certain cases increased solubility is accompanied with improved proteolytic stabilization (41,42). To conclude, the different strategies for stabilization presented in this review are valuable tools for evaluation and development of possible production strategies, but the method of choice is obviously depending on the inherent properties and final use of the target product.

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