Production of Antibody Fragments in *Escherichia coli*

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**INTRODUCTION**

As a result of the increasing commercial demand for antigen-binding antibody fragments for medical, industrial, and diagnostic applications, a need exists for a production process whereby these proteins may be produced efficiently and at low cost. Bacterial expression in, for example, *Escherichia coli*, where genetic manipulations and fermentation have been widely investigated, provides an alternative to animal cell culture. Large-scale processes for production of recombinant proteins from *E. coli* have been reported, even though in certain expression strategies where the recombinant protein is expressed in an insoluble form, bacterial expression may still be less economical.

Reviews of recent literature concerned with antibody engineering and the expression of antibody fragments in *E. coli* have already been published. Although antibody fragments have been expressed in an inactive form as inclusion bodies in the cytoplasm, a more common strategy is the direction of antibody fragments to the periplasmic space where folding to active antigen-binding proteins may occur.

Very few published reports to date have been concerned specifically with aspects of the fermentation of *E. coli* to produce high levels of functional antibody fragments. Nevertheless, it is clear that the expression of functional antibody fragments is affected by the nature of the expression-inducer system as well as the fermentation parameters. Much of this work has been performed in shake flasks where production levels have been low, but reports of concentrations of up to 450 mg/l in fermenter cultures of Fv fragments and 1 to 2 g/l of bivalent antibody fragments have been made.

This paper reviews recent literature on the production of antibody fragments by fermentation of *E. coli* with a view to integration with subsequent downstream processing. With this in mind, it is useful to relate the production of antibody fragments to more general information concerning recombinant protein production in *E. coli*.

**STRUCTURE OF ANTIBODIES AND ANTIBODY FRAGMENTS**

The antibody molecule consists of a Y-shaped tetramer of polypeptides consisting of two heavy and two light chains. (See Fig. 1.) The amino-terminal end provides variability (V) in both the heavy (H) and light (L) chains. These variable regions are known as $V_H$ and $V_L$, respectively, and together they form the antigen-binding site.
The remainder of the whole antibody consists of constant domains. The constant part of the light chain is known as CL, and the heavy chain constant section is further divided into three structurally discrete regions CH1, CH2, and CH3. All of the heavy and light chain regions are stabilized by intrachain disulfide bonds. Glycosylation usually occurs in the constant domains and is not involved in antigen binding; therefore, in the expression of recombinant antigen-binding fragments in organisms such as *E. coli*, which cannot perform this modification, there is no problem.

Essentially, three types of fragments containing the entire antigen-binding site have been successfully expressed from *E. coli*: Fab, Fv, and scFv. Fab may also be produced directly from the whole antibody by proteolytic cleavage using papain, and Fv can be produced by cleavage from Fab using pepsin, while the third, scFv (or single-chain Fv), requires the insertion of a polypeptide linker at the genetic level for production. These fragments contain the binding site of the whole antibody but are much smaller in size and cannot induce effector functions. Fv fragments consisting of the VL and VH domains only are the smallest fragments containing the complete antigen-binding site. Although antibody fragments can be produced by proteolysis, preparation of large quantities of Fv by this method would be costly.

![Diagram of antibody fragments](image)

**FIGURE 1.** Schematic representation of an antibody and derived antibody fragments.

Single-chain Fv antibody fragments consist of the VH domain and the VL domain connected in either order by a short polypeptide linker. Recombinant DNA technology allows this to be engineered in *E. coli* plasmid vectors and expressed as a single protein. The inclusion of a polypeptide linker in Fv fragments ensures expression of both domains in an equal ratio, may aid association of the immunoglobulin chains once translated, and will increase the stability of the fragment.

Calculations of the distance between the COOH-terminus of VH and the NH2-terminus of VL have determined that a 15-residue peptide would be sufficient. Computer searches of libraries of three-dimensional peptide structures and computer graphics were used to find suitable linkers that could join VH and VL of the variable regions of three different monoclonal antibodies by linkage of the COOH terminus of one domain to the NH2 terminus of the other. The secondary structure must not be so complex that it is difficult to fold or interferes with antigen binding;
however, if the linker is devoid of secondary structure, it may be susceptible to proteolysis. A number of linker polypeptides have been reported, with (Gly4Ser)15 being the most commonly used. Many linkers are known to reduce the affinity of the scFv for the antigen when compared with the Fab fragment; however, one linker is reported to slightly increase affinity. Folding and stability of scFv fragments has been investigated with three different linkers ranging from 12 to 25 amino acids in length.

APPLICATIONS OF ENGINEERED ANTIBODIES

Therapeutic uses concerned with tumor imaging and therapy appear to be the major target applications for recombinant antibody fragments with the global market predicted by CEST (UK Centre for Exploitation of Science and Technology) to be worth around $6 billion by the end of the century. The small size of Fv and scFv fragments are a characteristic that makes these particular proteins attractive for the basis of such products. Whole monoclonal antibodies have been shown, because of their large size, to be slow to reach the tissues to which they are targeted and subsequently have slow clearance times from blood and tissues. Fv and scFv fragments have been shown to overcome this problem and can easily be attached to radiolabels or toxins to enable imaging and treatment of tumors. Whole monoclonal antibodies may also attach nonspecifically to other tissues via the heavy-chain constant regions and, if developed in nonhuman cell lines, may elicit an immune response. This is also true of the smaller antibody fragments, but this may be overcome easily in fragments by using human framework and so humanizing the antibody. Alternative applications of smaller antigen-binding proteins such as Fv and scFv are in protein purification where the smaller size of the fragments allows immobilization to a greater capacity on porous supports, increasing the capacity of the column for the target antigen and also increasing the lifetime of the immunoaffinity column.

The presence of the linker also opens up more possibilities for applications of the fragment; for example, the linker may be used for the attachment of drugs or affinity handles or for immobilization to solid supports. The small size of these fragments is also an advantage and, with careful design of the linker, specificity of the binding site will be retained.

Finally, other alterations to the basic antibody fragments may be made to diversify their applications. Fusions of Fab fragments to enzymes have been reported; this will aid assay of recombinant proteins. The addition of histidine tails to aid purification of antibody fragments has also been engineered as well as bivalent mini-antibodies where two fragments having either the same or different specificities are fused together. Immunoliposomes have also been constructed in which the antibody fragment is lipid-tagged and may be reconstituted into liposomes.

TARGETING OF EXPRESSION TO CELLULAR LOCATIONS

Formation of Cytoplasmic Inclusion Bodies

The strategies for expression of recombinant antibody fragments in E. coli have already been extensively reviewed. The simplest method is by direct expression in the cytoplasm with no requirement for the use of signal sequences. Like other recombinant proteins, antibody fragments are susceptible to proteolytic degradation
by cytoplasmic proteases; therefore, high levels of expression are required to enable accumulation as insoluble and inactive inclusion bodies. Expression by this method has been demonstrated for whole-antibody heavy and light chains expressed from different plasmids both in separate cells and co-expressed from the same cell\textsuperscript{23,24} and for variable domains of both heavy and light chains expressed separately\textsuperscript{25,26}; however, recovery to active fragments was limited. \textit{E. coli} strains deficient in cytoplasmic proteases allowed accumulation to higher concentrations.\textsuperscript{28} Other antibody fragments that have been expressed as inclusion bodies include Fc fragments\textsuperscript{29} (see FIG. 1.) and scFv fragments.\textsuperscript{14,15} 

This, however, has the disadvantage of a requirement for an efficient refolding and renaturation process and, in the case of separate expression of the immunoglobulin chains, the formation of Fv heterodimer. Although this has been reported with some success by many groups, only one study has provided a fuller investigation\textsuperscript{31} whereby, considering the important parameters in the renaturation procedure, optimal conditions for the renaturation of Fab from inclusion bodies were determined.

Fusion to cytoplasmic proteins to accumulate increased concentrations of antibody fragments as inclusion bodies is also a possibility; however, specific cleavage of the fusion protein must be performed as well as renaturation to produce pure antibody fragments.

**Soluble Periplasmic Antibody Fragments**

A method leading directly to the production of functional assembled Fv fragments was developed by Skerra and Pluckthun.\textsuperscript{32} This mimics the eukaryotic folding and assembly pathway by assuming that the periplasmic space of \textit{E. coli} is equivalent to the lumen of the endoplasmic reticulum. The expression system of Skerra and Pluckthun used signal sequences of proteins naturally expressed in the periplasm of \textit{E. coli} to direct the antibody fragment to this location. The strategy of employing signal sequences for direction to the periplasm has also been reported for other recombinant proteins such as human growth hormone (hGH).\textsuperscript{33}

Extensive work on this expression system was carried out by Skerra and Pluckthun and their colleagues. They could successfully produce Fab fragments,\textsuperscript{6} scFv fragments,\textsuperscript{34} and related Fv fragments.\textsuperscript{35} Improvements to the vector allowing for better expression and engineering of the scFv by addition of histidine tail to allow for easy purification were also achieved.\textsuperscript{21}

A second strategy for functional expression of periplasmic antibody fragments has been demonstrated by Bregegere and Bedouelle,\textsuperscript{36} who fused scFv fragments to maltose-binding proteins and produced these fusion proteins at low temperatures (24°C).

The expression of functional Fab fragments secreted from the \textit{E. coli} cell to the culture medium has also been claimed.\textsuperscript{37} This was again achieved by the use of a leader peptide segment fused to immunoglobulin protein chains to direct them across the inner membrane of \textit{E. coli}. In this case 90\% of the functional Fab was located in the culture medium rather than in the periplasmic space.

**Expression on the External Cell Surface**

Fusion to outer membrane lipoproteins for successful expression of antibody fragments has also been recently reported.\textsuperscript{38} In this case 50,000 to 100,000 copies of
scFv per cell were produced as a fusion to lipoprotein and ompA. This expression method will require cleavage of the fusion to allow for pure scFv if utilized as a production method; however, it does provide an alternative to phage display for screening of libraries of scFv genes. Fusion of scFv to peptidoglycan-associated lipoprotein has shown reduced lysis of *E. coli* cells after induction compared to expression of free scFv.39

**PERIPLASMIC LOCATION**

Secretion to the periplasmic space of *E. coli* has a number of advantages. First, there are fewer proteases present in the periplasmic space compared with the cytoplasm, reducing the likelihood of proteolytic degradation. Even so, at least 8 out of the 25 known *E. coli* proteases are localized in this area; therefore, fermentation conditions optimized to reduce this action may be beneficial. This has not been studied directly for antibody fragments, but studies on *E. coli* producing other proteolytically sensitive recombinant proteins in the periplasmic space have shown that in addition to the use of protease-deficient mutant strains, low temperatures, acidic conditions, and the addition of Zn²⁺ ions may suppress protease action.40 Protease degradation was much greater at 42°C, and an explanation for this is that higher temperatures induce synthesis of proteases regulated by the heatshock response. The pH of the periplasmic space is related to the pH of the culture medium as small molecules of less than 600 Da can permeate the outer membrane so this may be easily regulated. Cytoplasmic pH, however, is maintained between 7.5 and 7.9. Protease action is maximized under alkaline conditions and inhibited at pH values of less than 6.0. The applicability of this approach to antibody fragment production is not yet known. Also, the effect of lower pH on stability and activity of antibody fragments remains to be investigated.

Folding to globular domains in the periplasmic space presents further resistance to enzymatic attack, and the oxidizing environment also allows for the formation of disulfide bonds. The periplasmic space is also the location for so-called folding catalysts, which aid the folding of proteins and formation of disulfide bonds.41 Knappik and co-workers42 suggested that the yield of functional antibody fragments produced in the periplasm may be limited by the folding process and the availability of folding catalysts. Attempts to alleviate this problem by overexpressing the folding catalysts resulted in no increase in functional expression. Misfolded proteins are either degraded by proteases present in the periplasm or are accumulated as inclusion bodies in the periplasm.

From a process viewpoint, secretion to the periplasm could have further benefits. Recombinant antibody fragments in this location are present in a small volume and in a highly concentrated form. If the periplasmic contents could be released specifically and efficiently, then purification would be straightforward. However, some leakiness of the outer membrane of *E. coli* has been observed during fermentation to produce antibody fragments. The biochemical basis of this is not really understood, but many parameters appear to be involved, such as host strain, plasmid, induction time, and temperature. Lysis of *E. coli* cells producing antibody fragments after induction for long periods of time at 37°C has been observed, because shortly after induction the outer membrane of the cells starts to become permeable.6 Leakiness does not appear to be related to the type of antibody fragment (Fv or Fab) or to the signal sequence used but more to the strain and growth conditions. Pack and his co-workers22 also concluded that leakiness of the outer membrane is directly related to the growth physiology. In the production of α-amylase, overexpression
resulted in damage to the outer membrane, and proteins were released by lysis. 

Nevertheless, when a strong inducible promoter was used, α-amylase was selectively released with no lysis of cells observed. Salt concentration has also been seen to have an effect on the outer membrane as cell lysis has been observed at low salt concentrations. Seo and co-workers suggested that a medium having a high osmolarity may be required to maintain the leaky outer membranes of recombinant cells intact. They also suggested that this could be the reason that their recombinant cells grew better in complex media such as LB than in nutrient broth.

**DEVELOPMENT OF FERMENTATION STRATEGIES**

Expression in batch fermentation conditions using a nutrient medium is commonly used to achieve initial expression of a range of antibody fragments of different sizes and specificities. Under these unoptimized fermentation conditions, titers of the order of 10 mg/l are common, and with improved fermentation strategy much higher titers are possible. A number of factors are reported to be highly influential in successful antibody fragment production, such as strain selection, batch/fed-batch fermentation, temperature, regulation of induction, and time of induced culture as well as protein engineering effects of the antibody fragments themselves.

**Batch and Fed-Batch Cultures**

The production of antibody fragments has been investigated in both batch cultures and high cell density fermentations. Batch fermentations generally result in low biomass concentrations, and after optimization of fermentation conditions the titers of antibody fragments reported from these fermentations have been variable from 40 mg/l up to 450 mg/l as a result of differences in factors such as promoter and medium.

The utilization of high cell density fermentation is an alternative route to increased concentration of engineered antibodies with levels of up to 1 to 2 g/l reported. A high cell density having OD550 nm of 120 to 150 resulted in up to 100 mg/l of antibody protein in the culture medium with titers of 1 to 2 g/l of functional protein associated with the cell that was released by sonication. Use of a mineral salts medium supplemented with digested casein, controlled carbon source feeding, and regulated induction were all crucial to high production levels. Using a defined medium and controlled fed-batch conditions, titers of 200 mg/l of active antibody have been reported. Here, fermentation was induced towards the end of the exponential/fed-batch phase when a level of biomass of around 20 g/l was achieved (although this increased up to around 40 g/l by the end of fermentation). In this system a basal expression level of antibody fragments was observed during the exponential phase; however, the maximal mini-antibody levels were achieved 4 hours after induction, and at this point growth became unbalanced. The product was found to be largely periplasmic, indicating that these culture conditions do not increase the permeability of the outer membrane. Shibui and colleagues also used a fed-batch method and defined culture medium for production of Fab fragments with the light and heavy chains being expressed separately and then combined. The recombinant proteins were also secreted to the periplasmic space where they accumulated in the form of inclusion bodies. Maximum titers of the light chain were 2.88 g/l and the heavy chain 1.28 g/l. Under optimal conditions the yield of functional fragments was 90 mg/l.
Fed-batch fermentation for the production of recombinant proteins in *E. coli* has been more generally reviewed.\(^{53}\) The success of this method depends on the utilization of feeding strategies for the supply of a growth-limiting nutrient (usually the carbon source) to minimize the production of metabolic by-products that can inhibit growth and product formation. Acetate formation is a common problem when glucose is supplied as a carbon source at high concentrations in the medium, and there are many reports of its inhibitory effects.\(^{54-56}\) A reduction in growth rate and protein production is observed at concentrations of 0.17 M acetate with complete inhibition at 0.23 M; these values are strain dependent.\(^{55}\) Acetate forms even if the dissolved oxygen concentration is still high, because the specific growth rate is increased when excess glucose is present. One solution is the use of alternative carbon sources such as glycerol that do not produce acetate. Another approach would be the control of specific growth rate by use of a feeding regime that provides carbon limitation. Reisenberg and co-workers describe the development of a medium\(^{57}\) and a control system\(^{58}\) that have allowed for high biomass and high-level expression of recombinant proteins in fed-batch fermentations. The glucose mineral medium reduces the number of feed streams to the fermentation. Only ammonia, which both controls pH and provides the nitrogen source, and glucose as the carbon source, which is fed in a limited manner to control specific growth rate and reduce inhibitory by-product formation, are required as feed streams. The use of low controlled specific growth rates between 0.1 and 0.15 hr\(^{-1}\) have also been reported to aid plasmid stability of recombinant *E. coli*.\(^{53}\)

**Medium Selection and Influences**

Media used for antibody fragments could be highly influential in the optimization of antibody fragment fermentations. Both complex and defined media have been employed, complex media more commonly in batch fermentation\(^{51,59,60}\) and defined media for fed-batch cultures.\(^{22,52}\) Complex media generally support higher specific growth rates; however, defined media in fed-batch fermentations have produced the highest cell concentrations. The use of complex medium components such as yeast extract to control growth rate during fed-batch cultures has resulted in higher specific concentrations of recombinant antibody fragments\(^{5,61}\) (mg/g) but low biomass.\(^{62}\) Higher titers of recombinant protein on complex media are probably due to the provision of amino acids. The use of digested casein in fermentation medium also gave particularly high titers of 1 to 2 g/l Fab in fed-batch fermentation.\(^{10}\)

Considering the high cell density strategy, the defined medium described by Reisenberg\(^{57,58}\) has been employed for antibody fragment fermentation\(^{22}\) as well as for the production of other recombinant proteins. Salt concentration also has an effect on growth, as specific growth rate is reduced below a 50 mM NaCl concentration and above 300 mM with complete inhibition at 400 mM in *E. coli* secreting recombinant proteins to the periplasm.\(^{44}\)

After controlled growth on a defined medium in a fed-batch fermentation, a second feed containing complex components such as yeast extract and bactotryptone, introduced at the time of induction, has been shown to improve the specific yield of recombinant products.\(^{63}\) The ratio of glucose to yeast extract in this phase has also been shown to be influential in that an appropriate level of glucose must still be supplied. Tsai and co-workers suggested that the effect of organic nitrogen during the induction period may be in the protection of the recombinant protein from proteolytic degradation. The metabolic effects of complex components in recombinant *E. coli* fermentation have been investigated in batch culture.\(^{64}\) Yeast extract has
been found to enhance growth and acetate utilization but has no effect on the stabilization of recombinant products. The latter effect, however, is observed on addition of peptone. The use of complex media for the initial batch phase of a fed-batch fermentation has been reported for production of recombinant hGH. In this instance, the fed-batch phase had two stages: first, the growth phase to high biomass was carbon limited by controlled glucose feed; this was followed by a second phase in which glucose feed was increased and phosphate in the medium was exhausted. It was reported that under these conditions biomass formation is blocked, but hGH is produced to high levels.

When complex media are utilized for batch fermentations, the inclusion of other components such as trace elements, thiamine, biotin, and salts were found to increase yields of antibody \( \text{V}_{\text{H}} \) domains in shake flask cultures when compared with growth on LB medium. For the production of recombinant \( \beta \)-galactosidase, however, the use of LB medium supplemented with yeast extract was found to increase concentrations of the enzyme in shake flask cultures more than by addition of other complex components such as tryptone and casamino acids. In fermenter cultures supplementation with yeast extract increased productivity by increasing both biomass and the specific enzyme production. These results were found to have some dependence on strain. Li and co-workers suggested that it was unlikely that the organic nitrogen source had a protective effect against protease action. They did speculate that yeast extract may provide components required for protein synthesis or may affect regulation of initiation of transcription and translation.

**Induction of Antibody Fragment Expression under Control of \( \text{lac} \) and \( \text{tac} \) Promoters**

The most commonly utilized expression systems for production of antibody fragments and also for other recombinant proteins from *E. coli* are the \( \text{lac} \) and \( \text{tac} \) promoter systems. Both are induced by addition of IPTG, and the wealth of information concerning the expression of antibody fragments from these promoters suggests that final concentration of IPTG and the temperature during the induced period as well as the time allowed for induction are strong influences on the titers of immunoglobulin proteins achieved and their location with respect to the *E. coli* cell.

Final concentrations of the order of 1 mM IPTG are common for the induction of recombinant genes placed under the control of \( \text{lac} \) and \( \text{tac} \) promoters. Using this inducer concentration titers of 1 g/l of total bivalent miniantibodies, of which 200 mg/l were active, have been produced. Other groups of workers have reduced IPTG concentration to 0.1 mM and observed increased titers. Shibui and Nagahara have investigated the effects of fermentation conditions and inducer concentrations required to produce high yields of Fab fragments in *E. coli*. In their expression system the immunoglobulin chains were directed to the periplasmic space under the control of the \( \text{tac} \) promoter, which is inducible by IPTG. Under standard conditions of 37°C and using an IPTG inducer concentration of 1 mM, the final concentration of Fab fragments was very low (less than 0.29 mg/l). Improved expression (4.5 mg/l) was achieved by lowering the IPTG concentration from 1 mM to 0.1 mM accompanied by a temperature shift from 37°C to 30°C upon addition of IPTG. McGregor and colleagues observed that a decrease in temperature during induction did not affect the yield of antibody fragments, but antigen binding was increased two- to threefold at the lower temperature.

For production of other recombinant proteins, Takagi et al. found that fermentation temperature and inducer concentration can have an effect on both protein
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folding in the periplasm and leakage to the culture medium in the production of recombinant subtilisin as well as increasing titers. Lower temperatures of 23°C and reduced IPTG concentrations avoided the formation of insoluble aggregates in the periplasm, and soluble proteins produced at this temperature were retained in this location. At 37°C and 2 mM IPTG concentration lysis occurred soon after induction.

Sommerville et al. found higher yields at 30°C than at 37°C for soluble scFv fragments and not much effect for cytoplasmic inclusion bodies. A loss of cell viability was observed after induction of the lac promoter with IPTG, and this was much greater at 37°C. A larger number of cells were present when the E. coli culture was grown at 30°C, and this could account for the increase in functional scFv. Although scFv was targeted to the periplasm, loss of cell viability after induction resulted in release of scFv to the culture supernatant and eventually cell lysis, which totally accounted for the appearance of scFv in the supernatant. The amount of correctly folded scFv also continued to increase after lysis. Production of scFv occurred without IPTG induction as a result of the leakiness of the lac promoter. Higher yields would be possible if a more tightly regulated promoter was used to reduce toxicity and allow growth to high cell densities.

Investigations into fermentation of E. coli for production of Fv fragments have shown that temperature shifts on induction with IPTG can have a profound effect on the location of the recombinant antibody fragment. A leader sequence directing the antibody fragment to the periplasmic space formed part of the expression system, and Fv fragments were retained in the periplasmic location if the temperature was increased from 25°C to 30°C upon induction; however, if the temperature was maintained at a constant 25°C the Fv was found largely in the culture supernatant. Investigations into the production of Fv fragments with different antigen-binding specificities also found that different fragments had different optimal temperatures for high production levels.

These two promoter systems have been directly compared, resulting in 20-fold higher production levels when expression is under control of the tac promoter. The high titers of 450 mg/l Fv fragments produced 12 hours after induction of a batch fermentation by addition of IPTG were from expression under the control of a tac promoter.

Shibui and Nagahara investigated the effects of time of fermentation on both yield and location of Fab fragments. Maximum yields were obtained 10 hours after induction. In the 10- to 22-hour period following induction, the concentration of Fab fragments in the periplasm fell and was accompanied by a concomitant increase in Fab concentration in the culture medium, clearly indicating that Fab fragments are released from the periplasmic space into the culture medium as the fermentation proceeds.

Takkinen et al. increased titers of their antibody fragments when cultures were grown at 30°C rather than 37°C. Also 6 hours after induction, cells started to lyse, and release of alkaline phosphatase to the culture medium was observed. After overnight induction the final concentration of antibody fragments was 5 mg/ml with 90% released to the culture medium.

Froyen et al. observed accumulation of Fv or scFv in the periplasm after induction with IPTG, reaching maximum levels 2 and 5 hours after incubation. Subsequently, a rapid decrease occurred along with leakage of fragments to the supernatant. Longer times of induction (18–20 hours) are required for maximal secretion in periplasm or supernatant.

Generally, the use of these promoters for antibody fragment expression has required low concentrations of the order of 0.05 to 1 mM of the expensive IPTG for
induction, low temperatures, and long periods of time after induction to enable high titers of antibody fragments to be produced.

**Alternative Promoter Systems**

Because of the leakiness of lac promoters allowing for auto induction and expression of antibody fragments before addition of IPTG, it has been suggested that more tightly regulated promoter systems may yield higher titers of recombinant immunoglobulin products. A number of alternative expression systems have been reported to successfully produce antibody fragments in *E. coli* fermentation, and these are discussed below.

Cheadle *et al.* expressed VH and VL separately under the T7 promoter and achieved 150 mg/l of inclusion bodies. Similar titers were achieved for production of scFv fragments. A number of groups have assembled the recombinant genes behind the T7 promoter and transformed the vector into a strain carrying T7 RNA polymerase, which is under the control of the lac promoter so that antibody fragments are produced after induction with IPTG.73-75 The effects of reduced IPTG concentration for induction and a decrease in temperature for the induction period were similar to those observed when the recombinant genes are placed directly under the control of the lac promoter; that is, the titers attained are increased.74 A comparison of this method of T7 induction with the use of a λpL promoter to induce T7 RNA polymerase after induction by increase in temperature to 42°C74 resulted in higher observed titers with an IPTG-induced system.

The direct placement of recombinant antibody fragment genes under control of λpL and λpR promoters has been investigated.14,16,65,76,77 Temperature-regulated induction of λpL promoter produced Fv fragments,77 and high levels of both VH and VL were observed after induction at 42°C. Johnson and Bird76 used the λpR promoter and induced by temperature shift from 30°C to 42°C. ScFv was expressed to maximum levels of 5 to 15% of total cell protein 15 to 30 minutes later with little further increase after this time. Use of both λpL and λpR placed in tandem demonstrated expression of VH domains65 after temperature induction. Soluble proteins were again directed to the periplasmic space and accumulated to a concentration of 30 mg/l. In this system, optimum conditions were controlled by the type of culture medium used, the age of bacteria at induction, and the induction time.

The tryptophan promoter was used in two early reports of recombinant antibody fragment production in *E. coli*26,27 and induced by addition of indoleacrylic acid. Investigations into optimisation of fermentation conditions60 reported that lowering temperature to 21°C or 30°C again resulted in higher yields of functional antibody fragments. In this case functional Fab was expressed in the *E. coli* cytoplasm, and yields were still very low, typically 0.1 mg/l. In addition the amounts of insoluble Fab were shown to increase with lower fermentation temperatures.

Better and Horwitz48 achieved high titers by using the araBAD promoter system from *Salmonella typhimurium*, which is tightly repressed before induction of *E. coli* cultures. Induction by addition of l-arabinose results in expression of antibody fragments that accumulate in the culture supernatant to high titers of 561 mg/l under controlled fed-batch fermentation conditions.

High titers were achieved by Carter *et al.*10 by use of *E. coli* alkaline phosphatase *phoA* promoter induced by phosphate starvation towards the end of exponential fed-batch growth. Tight control of expression was crucial in achieving high cell densities and high titers. A more recently investigated tightly controlled promoter for antibody fragment production is the tetracycline promoter Tn10TcR, which is
induced by addition of tetracycline or the less antibiotic anhydrotetracycline. Very similar yields and time course of fermentation was observed at 22°C to expression from a similar plasmid that uses LacUV5 promoter. In the latter case, some Fab was produced before induction, and toxicity was seen at 37°C. Expression from tetracycline promoter allowed for greater biomass in E. coli cultures and reduced toxicity. After 4 hours of induction at 22°C, a titer of 20 mg/l Fab was produced.

EFFECT OF PROTEIN ENGINEERING ON TITERS OF ANTIBODY FRAGMENT

The order of domains in scFv fragments is highly influential on the titers achieved with $V_L$-linker-$V_H$ in HyHEL10 anti-lysozyme antibody more highly expressed than $V_H$-linker-$V_L$. In the former orientation, titers of 3 to 5 mg/l in culture medium as well as 10 mg/l insoluble antibody fragment were produced. Anand et al. also found that placing $V_L$ upstream of the linker allowed for higher levels of scFv fragments and that secretion was dependent on domain orientation. In this investigation linker design also affected titers. Takkinen et al. utilized a flexible interdomain linker region of a fungal cellulase as the polypeptide linker between the two variable domains of an scFv. The yield achieved was 1 to 2 mg/l with efficient secretion and release. The fungal linker was compatible with secretion and gave no interference with assembly, stability, and function.

When producing Fab fragments, sequences on the constant domains were important. The production of two Fabs from different IgG subclasses were compared, and one gave increased expression. A deletion in $C_{H1}$ of the Fab that gave reduced titers resulted in proper assembly and comparable production levels to the first IgG. $C_{H1}$ domain largely determines the stability of a Fab fragment by the disulfide bond arrangement. Switching $C_L$ domains from kappa ($\kappa\kappa$) to lambda ($\lambda\lambda$) resulted in increased concentrations of soluble periplasmic Fab. Functional kappa chain formation in the periplasm was thought to be the limiting factor as very low titers of free light chain were observed. High titers of functional Fab are generally associated with excess light chain, and it is possible that increased titers of Fab are also due to more efficient disulfide bond formation with $\lambda\lambda$.

Humanized variable domains of Fab fragments resulted in increased titers and these humanized framework templates were used to display specificities against other antigens after E. coli expression to high titers.

STABILITY IN THE FERMENTER ENVIRONMENT

Glockshuber et al. observed instability of Fv fragments compared to Fab fragments when incubated at 37°C for extended periods. Three strategies, chemical cross-linking, an extra disulfide bridge, and production of scFv fragments, were compared, and all were found to increase the stability of the antibody fragment at 37°C. Cumber et al. also found that 40% of Fv activity had been lost over a 24-hour period of incubation at 37°C. However chemical cross-linking of two Fv fragments into a conjugate enabled 100% activity to be retained.

The fusion of Fv and scFv fragments directed against lysozyme to proteins and affinity tails has been reported to increase their stability in the fermentation environment. Both antigen binding and fusion protein functions were reported to be intact.
CONCLUSIONS

At present there is clearly no single, universal strategy in producing recombinant bacterial antibody fragments with a view to scale-up. This is not surprising, as many recombinant proteins have been shown to have highly system-specific requirements. It is clear that optimization of fermentation is required so that high titers of antibody fragments in a desirable cellular location, maintaining solubility, activity, and stability, are obtained. There is also a requirement for the impact of fermentation on subsequent downstream processing to be investigated. Optimal integration of these operations will be important in the elucidation of a complete and efficient process for antibody fragment production.

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