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REVIEW

Affinity Fusion Strategies for Detection, Purification, and Immobilization of Recombinant Proteins

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Genetic engineering enables the construction of gene fusions resulting in fusion proteins having the combined properties of the original gene products. Fusion can be made on either or both sides of the target gene depending on the specific application, but the majority of the described fusion protein systems place the protein of interest at the C-terminal end of a well-characterized fusion partner. Many different applications for fusion proteins in widespread areas of biotechnology have been reported (1–3), including a facilitated purification of the target protein, means to decrease proteolysis of the target protein, display of proteins on surfaces of cells and phage, and construction of reporter molecules for the monitoring of gene expression and protein localization as well as to increase the circulation half-life of protein therapeutics. However, as pioneered by Uhlén and co-workers in 1983 (4), the most frequent application of gene fusions has been for the purpose of affinity purification of recombinant proteins. This review focuses on the use of affinity tag fusions for detection, purification, and immobilization of recombinant proteins. A general overview of commonly used affinity tags is followed by some recent examples of specific applications for affinity tags and a discussion concerning possible future trends.

AFFINITY FUSION PARTNERS

There is a great interest in developing methods for fast and convenient purification of proteins. For example, to facilitate functional and structural studies of proteins derived from the rapidly growing number of genes coming out of genome programs such as the Human Genome Project (5), efficient and robust production and purification strategies are necessary (6). For industrial production of recombinant proteins, simple and fast purification methods introduced as an early unit operation can improve the overall economy of the process. A powerful purification technique made possible by the introduction of genetic engineering is to purify the target protein by the use of a genetically fused affinity fusion partner. Such fusion proteins can often be purified to near homogeneity from crude biological mixtures by a single, and fusion-partner-specific, affinity chromatography step.

To date, a large number of different gene fusion systems, involving fusion partners that range in size from one amino acid to whole proteins capable of selective interaction with a ligand immobilized onto a chromatography matrix, have been described. In such systems, different types of interactions, such as enzyme–substrate, bacterial receptor–serum protein, polyhistidines–metal ion, and antibody–antigen, have been utilized (7). The conditions for purification differ from system to system and the environment tolerated by the target protein is an important factor for deciding which affinity fusion partner to choose. In addition, other factors, including protein localization, costs for the affinity matrix and buffers, and the possibilities of removing the fusion partner by site-specific cleavage, should also be considered. Numerous gene fusion systems for affinity purification have been described in the literature, all with different characteristics (3,7–10). Some of the most commonly used systems are listed in Table 1, together with their respective elution condition(s) and agents suitable for specific detection of expressed fusion proteins.

The introduction of a recognition sequence for a chemical agent or a protease between the fusion partner and the target protein allows for site-specific cleavage of the fusion protein to remove the affinity fusion partner (see below). However, for many applications

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TABLE 1

Commonly Used Affinity Fusion Systems

Fusion partner	Size (kDa)	Ligand	Detection[a]	Elution[b]
Protein A (Pharmacia Biotech)	31	hIgG	Rabbit IgG–peroxidase conjugate	Low pH
Z (Pharmacia Biotech)	7	hIgG	Rabbit IgG–peroxidase conjugate	Low pH
ABP	5–25	HSA	HSA–FITC conjugate	Low pH
GST (Pharmacia Biotech)	26	Glutathione	Anti-GST antibodies	Reduced glutathione
poly His (Novagen; Qiagen)	~1	Me^{2+}-Chelator	Ni^{2+}–NTA–alkaline phosphatase conjugate Anti-H_6 antibodies	Imidazole/Low pH
MBP (New England Biolabs)	40	Amylose	Anti-MBP antiserum	Maltose
FLAG peptide (Kodak)	1	mAb M1 mAb M2	Anti-FLAG M2 mAb	EDTA/Low pH Low pH/FLAG peptide
PinPoint[c] (Promega)	13	Streptavidin/Avidin monomeric Avidin	Streptavidin conjugates	— Biotin

[a] Examples of commercially available reagents for detection.
[b] Most used elution method.
[c] A 13-kDa subunit of the transcarboxylase complex from *Propionibacterium shermanii* commercialized as the PinPoint System (Promega).

the affinity tag may be left on the target protein, e.g.: (i) to function as immunogens for the generation and purification of antibodies (6,11–13); (ii) when the analysis of the biological activity of the target protein is unaffected by the affinity fusion partner (14–23); (iii) for directed immobilization of the target protein (6,12,20,23–27).

In order to study different interactions between biomolecules, e.g., protein–protein, protein–DNA (RNA), it is indeed convenient to have the interacting protein immobilized in a directed manner via an affinity tag. This strategy has been employed when biomolecular interactions have been characterized in dedicated instruments, i.e., biosensors (27). Furthermore, immobilization of enzymes can be used to obtain bioreactors or to facilitate removal or reuse of the biocatalyst and to alter the characteristics of the immobilized enzyme or the intended reaction (23,28,29). Cysteine residues have been used to achieve directed immobilization (24,30), but this strategy has the drawback that added cysteines can be involved in the formation of unwanted intra- or intermolecular disulfides. Also polyhistidine and polyarginine tags have been utilized to achieve a directed immobilization (25,26), but additional interactions between the affinity matrices used (IMAC or ion-exchange resins) and the target protein might disturb the intended orientation of the immobilization. An interesting alternative is to use an *in vivo* biotinylated affinity fusion partner for immobilization of the fusion protein onto streptavidin- or avidin-coated surfaces (20, 27). The exceptionally strong interaction ($K_d \sim 10^{-15}$ M) between streptavidin and biotin ensures a robust and efficient immobilization. Furthermore, immobilization of bi- or multifunctional fusion proteins via one of its affinity activities is also a strategy for creating novel affinity matrices utilizing an immobilized target protein (20).

Staphylococcal Protein A and Its Derivative Z

Staphylococcal protein A (SPA) is an immunoglobulin-binding receptor present on the surface of the gram-positive bacterium *Staphylococcus aureus.* The strong and specific interaction between SPA and the constant part (Fc) of certain immunoglobulins (Ig) has made it useful for the purification and detection of immunoglobulins in a variety of different applications (31,32). SPA is capable of binding to the constant part of IgG from a large number of different species including man (31,33). In addition, SPA has the capacity to bind to a limited number of Fab fragments containing certain VH sequences (34,35). The cloning and sequencing of the SPA gene revealed a highly repetitive organization of the protein that could be divided into a signal sequence followed by five homologous domains (E, D, A, B, and C) and a cell surface anchoring sequence denoted XM (Fig. 1) (36,37). It was later shown that all five domains of SPA were separately capable of binding

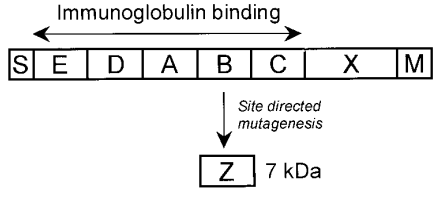

FIG. 1. Schematic representation of staphylococcal protein A (SPA) and the origin of the one-domain SPA analog Z. S, signal peptide (processed during secretion); E, D, A, B, and C, immunoglobulin-binding domains; XM, cell wall anchoring region.

IgG (38). The isolation of the gene made it possible to take advantage of different extensions of the receptor for use as affinity fusion partners, allowing one-step IgG-affinity purification of target proteins (Table 1) (4,39,40).

Several additional properties of SPA (or fragments thereof) have made it particularly suitable as fusion partner for production of recombinant proteins: (i) SPA is highly stable against proteolysis in various hosts (40). (ii) The N- and C-termini of the three-helix bundle structure of an individual IgG-binding SPA domain are solvent exposed (41–44), which favors an independent folding of a fused target protein and the SPA fusion partner; (iii) SPA does not contain any cysteine residues that could interfere with the disulfide formation within a fused target protein (36). (iv) SPA is highly soluble and renatures efficiently after having been subjected to denaturants such as urea and guanidinium-HCl, which facilitates the refolding of SPA fusion proteins from inclusion bodies or the "reshuffling" of misfolded or multimeric forms of disulfide-bridged target proteins (45–49). In addition, SPA fusion proteins can be produced to high levels within the *E. coli* cell and still remain soluble (6,18). (v) It has been demonstrated that it is possible to introduce different protease recognition sequences accessible for site-specific cleavage of SPA fusion proteins to release the target protein (48,50). (vi) The SPA fusion partner is secretion competent, and SPA fusions can be efficiently secreted to the *E. coli* periplasm and in some cases also to the culture medium, using the SPA promoter and signal sequence (51–55).

To allow chemical cleavage of SPA fusion proteins with both CNBr and hydroxylamine, an engineered domain based on domain B lacking methionine was designed. In this new domain, designated Z (Table 1), the glycine residue in a hydroxylamine sensitive Asn–Gly sequence was replaced by alanine, making it resistant to either of these chemical agents (Fig. 1) (56). In most of the recently constructed "SPA" affinity fusion vectors, constructs with different multiplicities of this Z domain have replaced the original SPA fusion partner. Analysis of different repeats of Z binding to IgG showed that there was no advantage in using more than two Z domains (24). A number of different expression vectors for *E. coli* production of Z or ZZ fusions in which the fusion protein either is exported (12,57–59) or kept intracellularly (6,18,48,49,60,61) have been developed. SPA and ZZ fusion proteins have also been successfully expressed in alternative hosts such as gram-positive bacteria, yeast, plant cells, Chinese hamster ovary (CHO) cells, and insect cells (40,62).

Albumin-Binding Protein from Streptococcal Protein G

Streptococcal protein G (SPG) is a bifunctional receptor present on the surface of certain strains of streptococci and is capable of binding to both IgG and serum albumin (63–65). The structure is highly repetitive with several structurally and functionally different domains (66,67). The regions responsible for the affinities to serum albumin and IgG, respectively, are structurally separated, which allowed for the subcloning of fragments displaying serum albumin binding only (Fig. 2) (65). The serum albumin binding region has been proposed to contain three binding motifs (ca. 5 kDa each), of which one has been structurally determined showing a three-helix bundle domain, surprisingly similar in structure to an IgG-binding domain of SPA (68,69). The region binds to serum albumins from different species with various affinities and shows strong binding to human serum albumin (HSA) (70). Expression in *E. coli* has demonstrated that the region shows a high proteolytic stability, is highly soluble, and can be efficiently secreted (6,12,65,71).

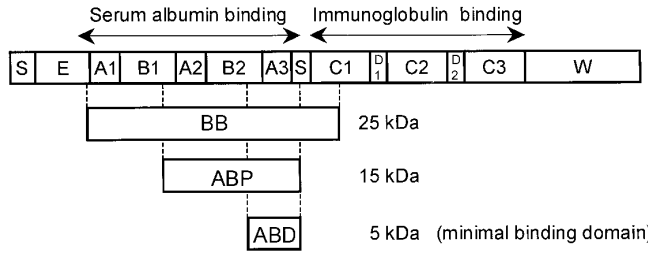

FIG. 2. Schematic representation of streptococcal protein G (SPG) and the extensions of various serum albumin-binding subfragments described in the text. S, signal peptide (processed during secretion); W, cell wall anchoring region. The regions responsible for serum albumin and immunoglobulin binding, respectively, are marked by arrows.

Different parts of the region, denoted BB, ABP (albumin binding protein) or ABD (albumin-binding domain) (Fig. 2) have been utilized as fusion partners, allowing one-step HSA-affinity chromatography purification of fusion proteins expressed either intracellularly or as secreted proteins (Table 1) (6,12,18,21,22,58,65,71–74). In addition to *E. coli*, other hosts such as COS and CHO cells (75) and gram-positive bacteria (76,77), have successfully been used to produce BB or ABP fusion proteins.

ABP and protein A (Z) fusion proteins are most conveniently eluted from affinity columns using low pH (e.g., acetic acid of pH $\approx$ 3). However, low pH can in some instances be destructive for target protein function and must in such cases be avoided. In addition, the use of low pH eluents following an initial capture of a fusion protein at physiological pH, could result in passage of the pH through the isoelectric point (p*I*) of the immobilized protein, which in some cases might lead to aggregation. Alternative elution conditions reported for ABP fusion proteins include high pH (pH 12) (75), lithium diiodosalicylate (0.25 M) (72), and heat

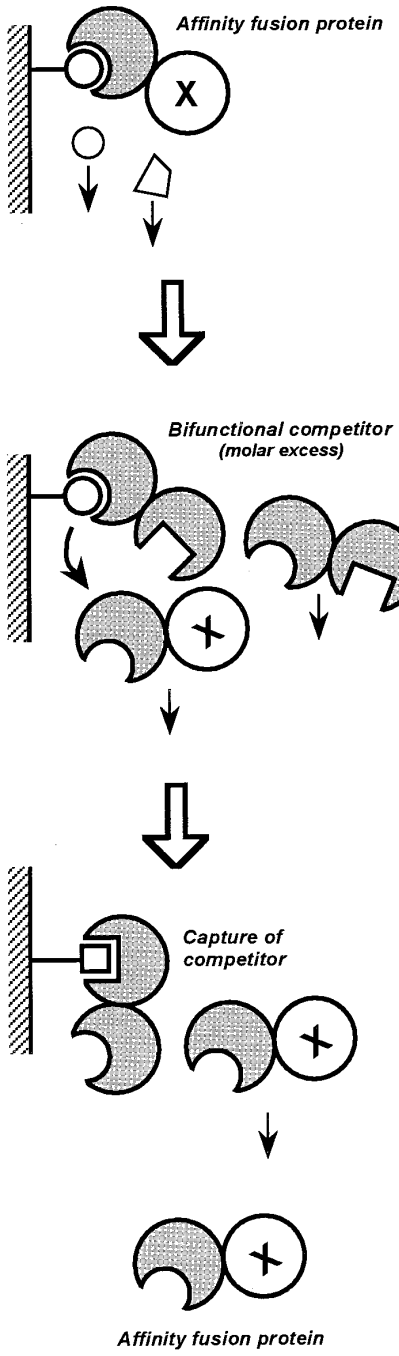

FIG. 3. Cartoon describing the concept of competitive elution using an engineered, bifunctional competitor protein.

(>70°C) (23). A general concept for mild elution of Z or ABD fusion proteins, based on competition, was recently devised (Fig. 3) (18). The target protein fused to a single Z domain is efficiently eluted by competition with a divalent ZZ competitor protein having a more than 10-fold higher apparent affinity for hIgG. The ZZ domains were fused to the divalent serum albumin binding region BB, allowing a means to specifically remove the competitor after elution via a second affinity function. Thus, the excess of competitor present in the eluate is captured through a simple HSA-affinity chromatography step. In addition, the strategy allows also for the reuse of the ZZ-BB competitor after elution of the fusion protein from the column. Furthermore, the ZZ-BB competitor should also be useful for effective competitive elution of monovalent albumin-binding domain fusion proteins.

The inherent properties of the serum albumin-binding fusion partners could make such fusion proteins of particular interest for delivery of protein therapeutics, as well as for administration of subunit vaccines. The strong and specific binding to serum albumin has been proposed to be responsible for the increased *in vivo* stability seen for otherwise rapidly cleared proteins (75,78). Serum albumin, which is the most abundant protein in serum, has a long half-life itself (19 days in humans) (79) and could therefore act as a "stabilizer" of sensitive proteins via a serum albumin-binding fusion partner. It has been demonstrated that the half-life in mice of a CD4 fragment (the target receptor for human immunodeficiency virus) could be significantly increased by fusion to BB (78). Likewise, fusion to different serum albumin-binding fragments of SpG was shown to increase the serum half-life of human soluble complement receptor type 1 (sCR1) in rats (75).

The serum albumin-binding region of SpG seems to have immunopotentiating properties, when used as a carrier protein genetically fused to a protein immunogen used for immunization (73). Recently, it was demonstrated that a BB fusion protein, BB-M3 containing a malaria peptide, M3, induced significant antibody responses in mice strains that were nonresponders to the malaria peptide alone or when fused to a different affinity fusion partner, suggesting that BB fusion partner has the ability to provide T-cell help for antibody production (80).

Polyhistidine Tags

In 1975 a new concept for affinity purification of proteins was presented by Porath and co-workers (81,82). The method is based on the interaction between the side chains of certain amino acids, particularly histidines, on a protein surface and immobilized transition metal ions, and is today known as immobilized metal ion affinity chromatography (IMAC). The metal ions are immobilized by the use of a chelating agent capable of presenting the metals for binding to the protein. Several gene fusion systems employing histidine-rich tags for purification of recombinant proteins on immobilized metal ions have since then been described (Table 1) (9). Tags, either N- or C-terminal, consisting of consecutive histidine residues binding selectively to immobilized

Ni^{2+} ions were described by Hochuli and co-workers (83). Adsorption of the poly-His-tagged proteins to a metal–chelate adsorbent was performed at neutral or slightly alkaline pH, at which the imidazole group of the histidines is not protonated (84). The expressed fusion proteins were recovered with a purity of more than 90% in a single step using a Ni^{2+}–nitrilotriacetic acid (NTA) adsorbent and elution with low pH or by competition using imidazole (83,84).

Other research groups have described the use of alternative chelators such as iminodiacetic acid (IDA) (27,85–88) or Talon (Clontech) as well as other His-containing tag sequences such as variants also containing Trp residues (85,89) or multiple copies of the peptide Ala–His–Gly–His–Arg–Pro (86). For IMAC purification of various His–Trp containing fusion proteins, the use of Co^{2+} or Zn^{2+} rather than Ni^{2+} as the immobilized metal ion has been shown to lead to less contamination of *E. coli* proteins (89). General expression vectors for both intracellular (27,87,90–93) and secreted production (94,95) of hexahistidine (His_6) fusion proteins in *E. coli* have been developed. Furthermore, His_6 sequences have also been employed in the production and purification of dual affinity fusions in *E. coli* in combination with a modified S-peptide of ribonuclease A (96,97), the albumin-binding protein ABP (22), or the GST fusion partner (98). The use of polyhistidines as fusion partners for purification has been demonstrated for recombinant fusion proteins produced in a wide range of host cells other than *E. coli: Saccharomyces cerevisiae* (13,99), mammalian cells (88,100), and baculovirus-infected *Spodoptera frugiperda* insect cells (101,102).

An important advantage of the His_6 affinity tag is the possibility of purifying proteins under denaturing conditions. Thus, proteins that have aggregated into inclusion bodies can be dissolved in a suitable agent such as urea or Gu-HCl and purified by IMAC. Refolding of the target protein can then be performed without interference from other proteins (84,103). Furthermore, small affinity tags such as the His_6 peptide can easily be genetically fused to a target gene by polymerase chain reaction (PCR) techniques (62,104). Also, the strong interaction between polyhistidine tags and immobilized Ni^{2+} ions has been demonstrated to result in a directed immobilization of the fused target protein, allowing protein–protein or protein–DNA interaction studies (25,90,105). However, when attached to recombinantly expressed DNA-binding proteins, histidine tails have been reported to influence the DNA-binding properties (106).

An interesting extension of the IMAC technology has been described by Lu and co-workers (107). Using the solubilizing *E. coli* thioredoxin protein as presentation scaffold, histidine residues were introduced at different positions that satisfied the geometrical constraints for metal ion binding. Such "histidine patch" thioredoxins could find broad use as fusion partners since they combine two beneficial features, high solubility and the possibility of using affinity chromatography for purification.

In Vivo Biotinylated Affinity Tags

The strong binding ($K_d \sim 10^{-15}$ M) between biotin and avidin or streptavidin (108–110) is frequently used in biochemistry and molecular biology for immobilization and detection purposes. For use in such applications, biotinylation of proteins is most frequently achieved through a covalent coupling of biotin to the protein by the use of biotin-ester reagents that preferentially modify lysine residues (111). However, such coupling is difficult to direct to a specific position and can occur at several residues, some of which might be important for the protein structure and/or activity. An elegant strategy for site-specific biotinylation of recombinant proteins during their production was demonstrated by Cronan, utilizing the *in vivo* biotinylation machinery of *E. coli* (112). Analysis of various extensions of the naturally biotinylated 1.3 S subunit of *Propionibacterium shermanii* transcarboxylase (113) showed that a 75-residue fragment was sufficient to serve as substrate for biotinylation. Thus, fusion proteins containing *in vivo* biotinylated domains could be purified by affinity chromatography employing immobilized monovalent avidin (114), and subsequently eluted with free biotin for mild elution (112). The stability of the avidin ligand also allows the capture of proteins under denaturing conditions, such as in the presence of 1% sodium dodecyl sulfate (SDS) or 8 M urea (112). Reusable matrices can also be obtained by chemical modification (nitration) of tyrosine in the biotin-binding sites of avidin and streptavidin. Such matrices called nitrostreptavidin or nitroavidin allow for elution of biotin-containing proteins by free biotin or alkaline pH (pH 10) (115).

Few proteins are naturally modified by biotin incorporation. In *E. coli* only one such protein, the biotin carboxyl carrier protein (BCCP) of acetyl-CoA carboxylase, has been found (116). The biotin is covalently attached to a specific lysine residue, a reaction catalyzed by biotin ligase (BirA) (112,113,117). The C-terminal 101 residues of the *E. coli* BCCP protein have been utilized successfully as an affinity handle for production and purification of a Fab antibody fragment in *E. coli* (20). The fusion protein was secreted into the culture medium and then purified on streptavidin agarose beads, resulting in a simultaneous directed immobilization of the antibody fragment allowing one-step immunoaffinity purification of a recombinant human tumor necrosis factor α (TNF) fusion protein. If desired, biotinylated proteins bound to streptavidin can be

eluted by boiling in an SDS–urea solution (118). In addition to the BCCP and the *P. shermanii* proteins, similar proteins from *Klebsiella pneumoniae* and *Arabidopsis thaliana* have been used to produce biotin-modified fusion proteins in *E. coli* (112,119). The 1.3 S subunit of *P. shermanii* transcarboxylase has also been described to function as a biotinylated fusion partner in recombinant Semliki Forest virus-infected baby hamster kidney (BHK) and CHO cells (120).

From a combinatorial library of short peptides, sequences capable of mimicking the normal BirA substrate were found using an elegant selection strategy based on fusion of the peptides to a plasmid binding *lac* repressor protein (121). A family of sequences (ca. 13 aa) were found to be *in vivo* biotinylated at a central invariant lysine residue and could replace the larger *in vivo* biotinylated fusion partners used earlier by other groups. Recently, such short tags have found use in different purification and immobilization situations (27,122).

An alternative small affinity tag (nonbiotinylated) that binds to streptavidin has been found in a peptide library screened for peptides that mimic biotin (123). A nine-amino-acid streptavidin-binding peptide termed strep-tag was selected from the library and used as a C-terminal affinity fusion partner for purification of an *E. coli* expressed antibody Fv fragment by streptavidin-affinity chromatography. The fusion protein was competitively eluted with the biotin analog iminobiotin, which allowed for column regeneration by washing with the equilibration buffer. Such mild elution conditions (diaminobiotin) have also been used in the streptavidin-affinity chromatography purification of various membrane proteins captured via strep-tag fused Fv fragments (124). Recently, an improved version of the peptide, strep-tag II, was described (125). In contrast to the original peptide, strep-tag II has the potential to retain its streptavidin-binding activity when placed at positions other than the C-terminus.

A possible complication associated with avidin/streptavidin-affinity chromatography of *in vivo* biotinylated proteins is that any free biotin should preferentially be removed before loading the cell lysate onto the column, to avoid blocking of the ligand. In addition, if not removed in a first purification step, biotinylated host proteins may be copurified with the biotinylated target protein. Recently, this problem was circumvented using a combined affinity tag that enabled removal of biotin and BCCP by employing a first HSA-affinity chromatography step prior to immobilization of the affinity fusion protein onto a streptavidin surface (27). A similar approach was described for the production of a BCCP-firefly luciferase fusion protein in which a His_6 tag was also included for IMAC purification, prior to immobilization of the fusion protein onto avidin-coated surfaces (126). To increase the fraction of biotinylated recombinant proteins using BCCP or shorter tags as fusion partners, coexpression of the biotin ligase and/or addition of biotin to the growth medium have proven efficient (20,122).

Glutathione S-Transferase

The glutathione *S*-transferases (GST) are a family of enzymes that can transfer sulfur from glutathione to substances such as nitro and halogenated compounds, leading to their detoxication (127). Many mammalian GSTs can be purified by affinity chromatography using the immobilized cofactor glutathione, followed by competitive elution with reduced glutathione (128). Based on this specific interaction, a gene fusion system for *E. coli* expression was developed by Smith and co-workers (129), using GST from the parasitic helminth *Schistosoma japonicum* (Table 1). They demonstrated that a number of eukaryotic proteins fused to the C-terminus of GST could efficiently be purified from a crude *E. coli* lysate by glutathione-affinity chromatography. In addition, cleavage sites for the proteases thrombin and blood coagulation factor Xa had been introduced to allow removal of the GST fusion partner (129). It has been demonstrated that GST fusion proteins can be renaturated from inclusion bodies after solubilization using 6 M Gu-HCl and subsequently purified by glutathione-affinity chromatography (130). Recently, a series of vectors was described allowing either N- or C-terminal fusion of a target protein to GST for production in *E. coli* (131). Furthermore, two vectors have been developed that allow, in addition to N- or C-terminal fusion to GST, site-specific *in vitro* ^{32}P labeling of the fusion protein after glutathione-affinity purification, employing a protein kinase recognizing an introduced substrate sequence (132). The GST fusion partner has also been used as an N-terminal constituent in a dual affinity fusion approach, in combination with a His_6 tag at the C-terminus of a tripartite fusion protein (98). Furthermore, expression vectors have been constructed for production of GST fusion proteins in insect cells, S. *cerevisiae*, and COS-7 cells. (133–136). A possible complication associated with the GST fusion system is the use of reduced glutathione (a reducing agent) for elution, which can affect target proteins containing disulfides (137).

FLAG Peptide

An affinity gene fusion system that has become popular in recent years is the so-called FLAG system, based on the fusion of an eight-amino-acid peptide to the target protein for immunoaffinity chromatography on immobilized monoclonal antibodies. The FLAG peptide sequence (Asp–Tyr–Lys–Asp–Asp–Asp–Asp–Lys) is hydrophilic and contains an internal enterokinase cleavage recognition sequence (Asp–Asp–Asp–

Asp–Lys) (138). Monoclonal antibodies to the FLAG sequence were obtained after immunization of mice with a FLAG fusion protein. One monoclonal antibody (M1) was found to bind to FLAG fusion proteins in a calcium-dependent manner, which allowed gentle elution by the addition of a chelating agent (138,139). A limitation of the M1 antibody is that it can only bind the FLAG peptide when located at the extreme N-terminus of the fusion protein. This limits the use of the M1 antibody to the purification of fusions exposing an N-terminal FLAG peptide, such as after removal of a signal peptide from a secreted FLAG fusion. Recently, it was found that the M1 antibody binds with almost the same affinity to a shortened version of the FLAG peptide, consisting only of the first four amino acids (Asp–Tyr–Lys–Asp) (140). Alternatively, a different monoclonal antibody (M2), capable of binding to the FLAG peptide also if it is preceded by a methionine at the N-terminus or fused to the C-terminus of the target protein, is available (141). However, the M2 antibody interacts with the FLAG peptide in a non-calcium-dependent manner and a fusion protein can therefore not be eluted with a chelating agent. Instead, low pH or competition with an excess of synthetically produced FLAG peptide is used (Table 1) (141). Different hosts have been used for production of FLAG fusion proteins, including *E. coli*, *S. cerevisiae* (138), recombinant baculovirus-infected *S. frugiperda* insect cells (101), and COS-7 cells (142).

Maltose-Binding Protein

E. coli maltose-binding protein (MBP), the product of the *mal* E gene, is exported to the periplasmic space, where it binds specifically with high affinity to maltose or maltodextrins for subsequent transport of these sugars across the cytoplasmic membrane (143). MBP can be purified in a single step by affinity chromatography on resins containing cross-linked amylose followed by competitive elution with maltose (144). This fact together with the low cost for amylose resins, mild purification conditions, and the fact that MBP does not contain any cysteine residues that can interfere with disulfide bond formation within the target protein led to the development of an expression system for MBP fusion proteins in *E. coli* allowing affinity purification under mild conditions (Table 1) (145). Vectors for both intracellular and secreted production have been developed (14,145). The vectors designed by Guan and coworkers were further developed by the introduction of a cleavage recognition sequence for the factor Xa protease at the C-terminus of MBP to allow release of a target protein fused to MBP (146). Recently, expression vectors allowing translational fusions to MBP in all reading frames were presented (147). The possibility of renaturing an MBP fusion protein from inclusion bodies followed by amylose-affinity purification has been demonstrated (148).

SITE-SPECIFIC CLEAVAGE OF FUSION PROTEINS

For certain applications it is necessary to remove the affinity fusion partner after purification. For example, a fusion partner can cause unwanted immune responses, alter the properties, or complicate a structural determination of a fused target protein. Several methods have been described for site-specific cleavage of fusion proteins based on chemical or enzymatic treatment of the fusion protein (9,149) and some relevant examples are listed in Table 2.

Chemical cleavage methods are relatively inexpensive, but the rather harsh reaction conditions may lead to protein denaturation or amino acid side chain modifications of the target protein. Furthermore, the chemicals are relatively unspecific, which can lead to unwanted cleavages inside the target protein (149). Enzymatic methods are preferred to chemical methods because they are generally more specific and the cleavage can usually be performed under mild conditions (Table 2) (50,149).

After cleavage, the released affinity tag can be captured by passage of the cleavage mixture over an affinity matrix while the target protein is obtained in the flowthrough. However, in such schemes the protease is still left with the target protein and must be removed to obtain a pure target protein. To circumvent this, affinity-tagging of the protease has been employed in order to easily remove the protease after cleavage (see below) (19,22,153).

SPECIFIC EXAMPLES AND POSSIBLE FUTURE TRENDS OF AFFINITY TAG APPLICATIONS

Combined Affinity Tags

A common problem in heterologous gene expression is proteolytic degradation. Using an affinity fusion strategy, eventual degradation products of the target protein are copurified with the full-length fusion protein. Gene fusions can in some cases be used to stabilize labile proteins, but single fusions have shown limited stabilizing effects (154). However, it has been observed that dual affinity approaches can have a stabilizing effect on several proteolytically sensitive proteins compared to single fusions (154). Employing a dual affinity approach, as first described for the production of the peptide hormone insulin-like growth factor II (58), two different affinity fusion partners are fused at each end of the target protein. This allows for two successive affinity purification steps to obtain proteins that by definition only contain both tags and therefore also the central target protein (Fig. 4) (58). Several other combinations of affinity tags have been used in various dual affinity

TABLE 2

Examples of Methods for Site-Specific Cleavage of Affinity Fusion Proteins

Cleavage agent	Fusion protein	Cleavage specificity[a]	Cleavage conditions	Reference
Chemical agents				
CNBr	β-gal-somatostatin	-XM ↓ X-	70% formic acid, RT	150
Hydroxylamine	ZZ-IGF-I	-XN ↓ GX-	pH 9, 45°C	53
Enzymes				
Enterokinase	FLAG–interleukin-2	-X$(D)_4$K ↓ X-	pH 8.0, 37°C	138
Factor Xa	MBP–paramyosin	-XIEGR ↓ X-	pH 7.2, RT	146
Thrombin	GST–Ag63	-XLVPR ↓ GSX-	pH 8.0, 25°C	129
H64A subtilisin	ZZ–PTH	-XFAHY ↓ X-	pH 8.6, 37°C	151
IgA protease	MS2–His_6–F_V	-XPAPRPP ↓ TX-	pH 7.4, 37°C	152
GST[b]–protease 3C	His_6–PKCI	-XLETLFQ ↓ GX-	pH 8.2, 4°C	19
ABP–protease 3C–His_6	ABP–ΔTaq	-XLEALFQ ↓ GPX-	pH 8.0, RT	22

Note. Abbreviations used: CNBr, cyanogen bromide; β-gal, β-galactosidase; IGF-I, human insulin-like growth factor I; PTH, human parathyroid hormone; MS2, bacteriophage MS2 polymerase; PKCI, protein kinase C inhibitor; RT, room temperature.

[a] X = unspecific amino acid.

[b] Also His_6–protease 3C.

fusion concepts (97,98,122,155). Thus, the dual affinity approach points out the advantage, for certain applications, of using a combination of different affinity tags.

Although a multitude of systems have been described, no single affinity fusion strategy is ideal for all expression or purification situations. For example, a fusion to affinity tags derived from normally intracellular proteins (e.g., the β-galactosidase system) is not applicable when secretion of the product into the periplasm or culture medium is desired. Further, if the purification has to be performed under denaturating conditions, protein ligands such as monoclonal antibodies (mAbs; e.g., anti-FLAG M1 and M2 mAbs) are likely to be unsuitable. Instead, a combination of affinity fusion domains could be introduced into a single fusion partner to obtain general expression vectors for affinity gene fusion strategies, applicable in several situations. Such composite fusion partners consisting of several independent affinity domains could potentially be used for different detection, purification, and immobilization purposes, employing the affinity function most suitable for the situation. However, the affinity domains included should be carefully chosen so that they do not functionally interfere with each other, and each moiety should be able to withstand the purification conditions dictated by the affinity domain requiring the harshest affinity chromatography conditions. For instance, if denaturating conditions are used, as in IMAC purification of proteins containing, e.g., a hexahistidine sequence solubilized from inclusion bodies by urea or Gua-HCl, the other included affinity domains should not be irreversibly denatured during this step, which otherwise may cause aggregation in subsequent renaturation steps. A further advantage of using combined affinity tags is that different methods may be used for the detection and immobilization of the fusion protein.

Recently, a new affinity fusion partner based on the concept of combined affinity tags was described (27). A general expression vector for N-terminal fusion to an affinity tag combination consisting of an *in vivo* biotinylated sequence (Bio), a hexahistidine (His_6) sequence, and the albumin-binding protein (ABP) was constructed (27). Fusion to the Bio–His_6–ABP affinity tag allows the use of several commercial agents for detection, the possibility of purification under both native

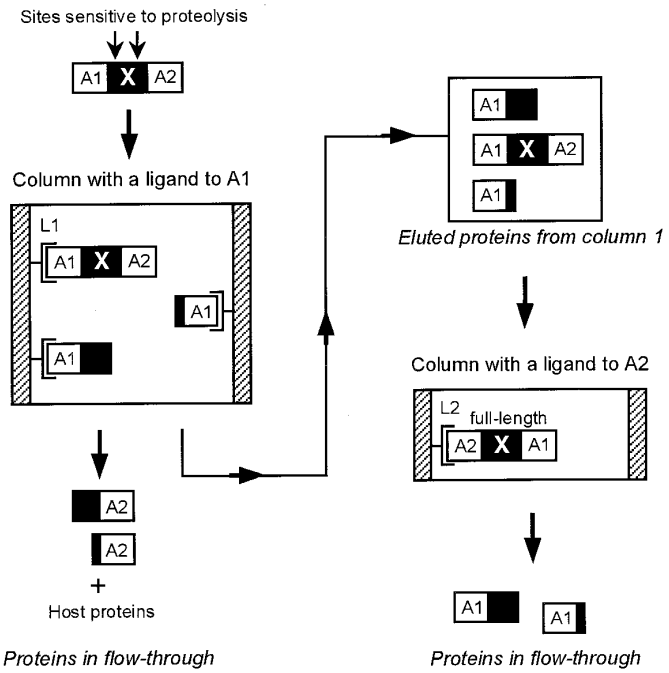

FIG. 4. The dual affinity fusion concept. The target protein is produced as flanked by two different affinity domains and purified using two consecutive affinity chromatography steps.

and denaturating conditions, and an option to choose from three strong affinity interactions for immobilization. In addition, the ABP part of the affinity tag is soluble and effectively refolded after denaturation, which can be an advantage during an eventual refolding of the fused target protein. The use of this particular combination of affinity tags also demonstrates the advantage of consecutive affinity purification steps for a certain application. Using the ABP–HSA interaction as the first purification, removal of biotinylated host proteins and biotin was possible. This allowed for immobilization of the Bio–His_6–ABP affinity tag onto a streptavidin chip in a Biacore instrument for biospecific analysis in the next step. Consecutive affinity domains have also been used as C-terminal fusion partners for the evaluation of the best affinity purification method for a rat neurotensin receptor (122).

Affinity-Tagged Proteases for Site-Specific Cleavage

In gene fusion strategies for the production of native proteins, efficient means for site-specific cleavage of the fusion protein and subsequent removal of the affinity fusion partner are needed. In addition, when enzymatic strategies for cleavage are employed, strategies for the removal of the protease itself must be developed. Special consideration must be taken if the target protein itself has to be further processed to give the desired final product (48,49). Careful upstream design of the fusion protein construct using genetic strategies can greatly facilitate the subsequent purification of the target protein and also allow for integrated systems involving coprocessing of the protein and efficient removal of the affinity fusion partner as well as the protease used for cleavage (22,49).

For general use in site-specific cleavage of fusion proteins comprising relatively large target proteins, proteases such as trypsin, which recognizes basic arginine and lysine residues, are not ideal, because these amino acids generally occur in proportion to protein size. Alternative enzymes for broader use in biotechnological applications should preferably be highly specific proteases, easy to produce by recombinant means in large scale. Interesting candidate enzymes can be found in human picornaviruses, whose maturation relies on the site-specific cleavage of a large polyprotein precursor to yield the viral components. Some of these proteases have been described to be functionally produced at high levels in bacterial expression systems (19,156), which also would facilitate the production of variants constructed by protein engineering. For example, a kit containing the 3C protease of rhinovirus (Table 2) fused to an affinity tag (GST) to be used for specific cleavage of target GST-affinity fusion proteins was recently presented (19, PreScission system, Pharmacia Biotech). This enables removal of the affinity-tagged protease

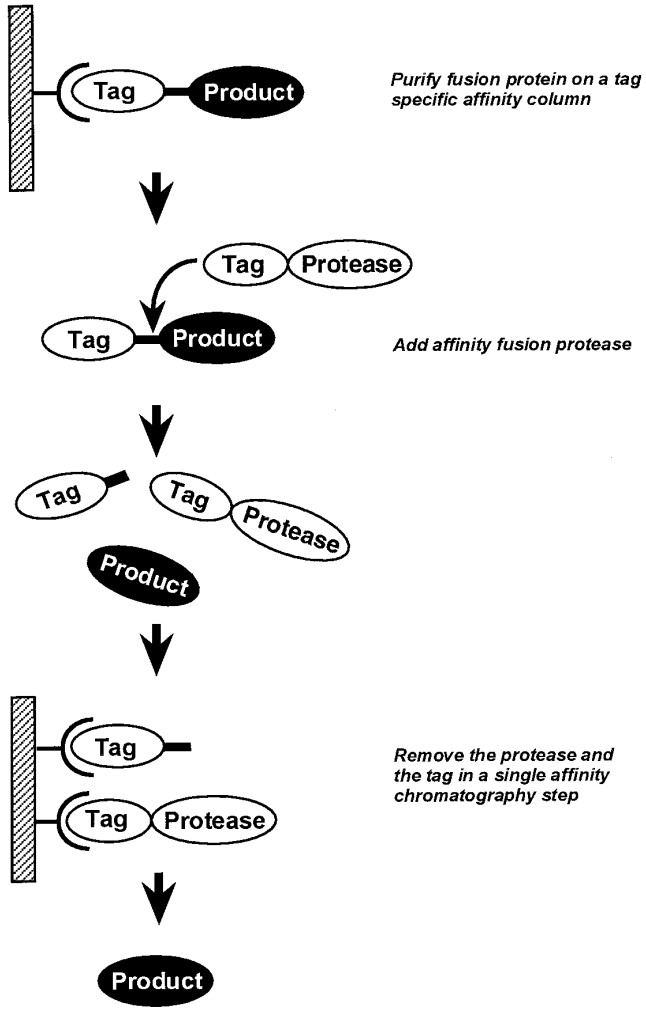

FIG. 5. The affinity fusion protease concept used in the purification of Δ*Taq* DNA polymerase.

and the released affinity fusion partner by simple affinity chromatography steps. In addition, the desired amount of protease to ensure efficient cleavage may be added, since the protease is easily removed after cleavage.

Recently, a different affinity-tagged protease, consisting of coxsackievirus 3C ($3C^{pro}$) fused to the serum albumin-binding ABP at the N-terminus and His_6 at the C-terminus (Table 2), was produced in *E. coli* and used for the production of a truncated *Taq* DNA polymerase (Δ*Taq*), according to the principle described in Fig. 5 (22). The heat-stable polymerase was produced as an ABP–Δ*Taq* fusion protein having a $3C^{pro}$ cleavage site introduced between the two protein moieties. After HSA-affinity purification and heat elution of ABP–Δ*Taq*, the fusion was cleaved using the affinity-tagged protease ABP–$3C^{pro}$–His_6, which allowed for the recovery of nonfused, fully active Δ*Taq* after passage of the cleavage mixture over an HSA column (22).

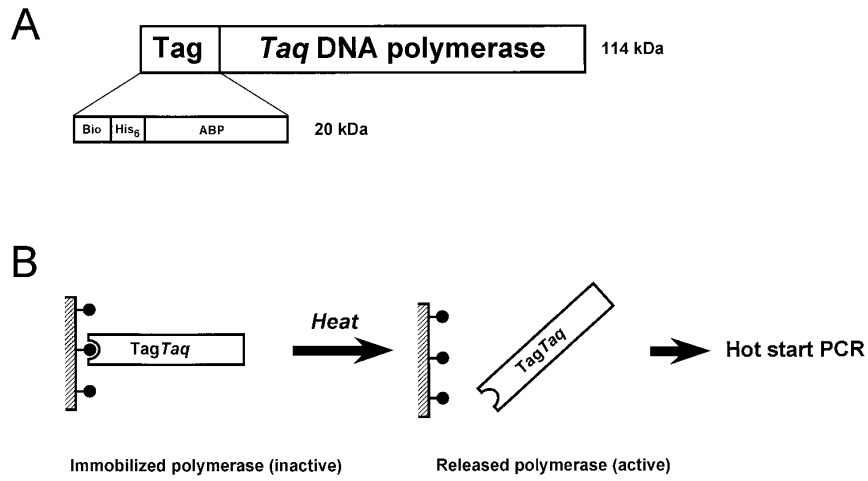

FIG. 6. Schematic description of the affinity immobilization strategy for *Taq* DNA polymerase used to achieve hot-start PCR. (A) The TagTaq fusion protein containing *Taq* DNA polymerase fused to the Bio–His_6–ABP affinity tag. (B) The principle for reactivation of the inactive, immobilized TagTaq fusion protein through heat-mediated release from the affinity resin.

Furthermore, an affinity fusion protease can also facilitate the on-column cleavage of an affinity fusion protein immobilized onto an affinity column in cases in which the affinity ligand is insensitive to the protease (19,22). Recently, a novel system for simultaneous affinity purification and on-column cleavage of affinity fusion proteins was described (157,158; Impact I System, New England Biolabs). In this system the target protein is fused to a fusion partner consisting of a modified protein splicing element (intein domain) from *S. cerevisiae* fused to a chitin-binding affinity tag. After a one-step affinity immobilization of the fusion protein on a chitin-containing column, free target protein can be eluted after induction (+4°C, reducing conditions) of the specific proteolytic activity of the intein domain, which itself remains immobilized via the chitin-binding domain (on-column cleavage).

Heat-Mediated Activation of an Affinity-Immobilized Taq DNA Polymerase

Immobilization of enzymes has proven to be a useful procedure for many applications, due to altered characteristics of the immobilized enzyme compared to its soluble counterpart (28). One practical application where such differential activities are utilized was recently described in which a heat-mediated release of an affinity-immobilized recombinant *Taq* DNA polymerase (TagTaq) was used to create a so-called hot-start PCR procedure (23). *Taq* DNA polymerase was produced in *E. coli* as fused to the multifunctional Bio–His_6–ABP tag (see above and Ref. (27)) for affinity purification and immobilization. HSA-affinity immobilization of the fusion employing the ABP moiety resulted in a deactivation of the *Taq* DNA polymerase (Fig. 6). However, the ABP–HSA interaction was shown to be disrupted at elevated temperatures (>70°C), resulting in a restored DNA polymerase activity of released *Taq* DNA polymerase fusion protein. This concept for controlling the activity of the *Taq* DNA polymerase was demonstrated to be of practical use to achieve a hot-start PCR procedure when it is desired to suppress the DNA polymerase activity until temperatures at which unspecific annealing of PCR primers is eliminated are reached. The affinity-immobilization concept for hot-start PCR was successfully used in full multiple-cycle PCR amplification and was shown to eliminate artifactual primer–dimer products in the amplification of the human oncogene K-ras gene, in contrast to standard amplification protocols. Noteworthy was that the ABP-affinity fusion partner was still functional after 30 PCR cycles, allowing post-PCR reimmobilization of the *Taq* DNA polymerase fusion protein onto fresh HSA–Sepharose and facilitating the removal of the DNA polymerase from reaction mixtures after PCR. Further studies are needed to determine if this concept for modulating biological activity can also be applied by using other tags and for other widely used enzymes (e.g., reverse transcriptase).

Affinity-Assisted in Vivo Folding

A new concept for improving the fraction of correctly folded recombinant insulin-like growth factor I (IGF-I) was recently presented (21). It was demonstrated that coexpression of a specific binding protein, IGF-binding protein type 1 (IGFBP-1), significantly increased the relative yield of IGF-I having native disulfide bridges when expressed in a secreted form in *E. coli*. In addition, a glutathione redox buffer was added to the

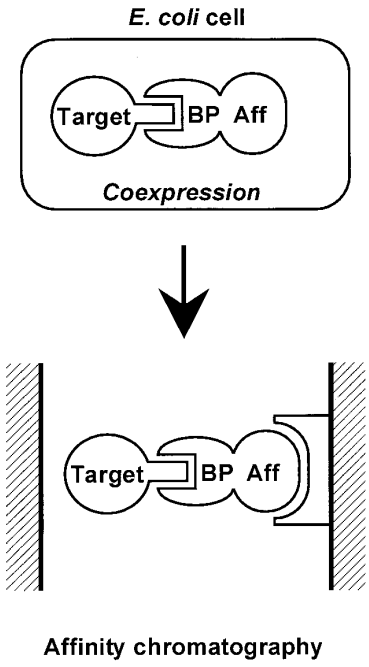

FIG. 7. The principle of affinity assisted folding used to improve the yield of correctly folded recombinant IGF-I in *E. coli* via coexpression of an affinity-tagged IGF-I specific binding protein. The affinity tag was subsequently used for efficient and selective recovery of the native folding form of the target protein.

growth medium to enhance formation and breakage of disulfide bonds in the periplasm of the bacteria. In the presented example, both IGF-I and IGFBP-1 were produced as affinity fusions, which facilitated the purification of *in vivo* assembled heterodimers by different methods (21).

For general use of the concept, an attractive strategy would be to express the target protein as a nonfused gene product and the specific binding protein in an affinity-tagged configuration (Fig. 7). Correctly folded target protein would thus be affinity captured as a heterodimer via the tagged binding protein. This would employ the benefits of high specificity for affinity chromatography without introducing requirements of proteolytic processing to recover the native target protein. The binding proteins to be used in these kind of bioprocesses could be naturally existing ligands, as in the described example (21), or optionally, novel proteins specific for correctly folded molecules selected from protein libraries produced employing combinatorial protein chemistry.

CONCLUDING REMARKS

During the past decade, affinity fusion technology has become increasingly important in many fields of research, including biochemistry, molecular biology, applied microbiology, immunotechnology, vaccinology, and biotechnology. Affinity fusion tags have been used as research tools for detection, purification, and immobilization of recombinant proteins expressed in bacteria, yeast, insect cells, plants, and mammalian cells. However, on an industrial scale the use of affinity fusions to facilitate bioprocessing of recombinant proteins has not yet been widely performed. The main reason for this is most likely due to the fact that there has been a demand to produce heterologous proteins in a native form in the "first generation" of recombinant products. Affinity fusion strategies thus introduce an additional problem into the downstream processing, since a site-specific cleavage is needed. This review points out new strategies based on affinity fusions, such as the use of affinity-tagged proteases and affinity assisted *in vivo* folding, which would facilitate downstream bioprocessing by circumventing some of the problems related to proteolytic cleavage of fusion proteins, and thus decrease overall production costs. Such strategies could potentially be implemented in the biopharmaceutical industry in the near future. For protein-level characterization of the immense number of genes or gene fragments emerging from various genome projects, affinity fusion strategies could facilitate automated handling of small-scale protein purification from cell lysates using established 96-well formats (159). We believe that the applications described in this review make it evident that affinity fusion strategies will find even broader use in the near future.

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