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ABSTRACT

Proteases are enzymes that cleave proteins. They occur widely in nature and serve a fundamental role in cellular homeostasis; however, their presence can result in unwanted protein degradation during recombinant protein expression and purification. This unit introduces proteases, specifically outlining the types commonly encountered during production of recombinant proteins. The strategies used to avoid and to prevent proteolysis are also highlighted with extensive consideration of the molecular, technical, and logical methodologies involved. *Curr. Protoc. Protein Sci.* 71:5.25.1-5.25.7. © 2013 by John Wiley & Sons, Inc.

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INTRODUCTION

Proteases are a class of enzymes that occupy a central position with respect to their physiological roles as well as their impact on biotechnology. The general reaction catalyzed by proteases is the degradation (or breakdown) of proteins. The importance of proteases is illustrated by the fact that they are found in all forms of living organisms. All cells maintain a rate of protein turnover by continuous degradation and synthesis of proteins. In addition, many cells produce extracellular proteases that break down large proteins into smaller proteins for absorption (see ?Rao et al., 1998? for review). All these protease activities have the potential to damage recombinant proteins during their expression. In this unit, the strategies used to avoid and prevent proteolysis during the expression of recombinant proteins are examined.

Proteases are members of the hydrolase family of enzymes (EC: 3.4). This enzyme family hydrolyzes peptide bonds with the participation of a water molecule. Proteases can be classified based on where this cleavage takes place. If the cleavage takes place within the polypeptide backbone, the protease is referred to as an endoprotease. Examples of endoproteases include trypsin and pepsin. Alternatively, if the cleavage takes place at the end of the polypeptide backbone, the protease is referred to as an exoprotease, with some common examples being aminopeptidases and carboxypeptidases. Proteases have specific cleavage sites; for example, trypsin cleaves proteins at the carboxyl side of the amino acids lysine and arginine.

The most common recombinant host for protein production, *E. coli*, is known to possess both endo- and exoproteases distributed throughout the cell (Makrides, 1996; Huang et al., 2012). Mammalian cells contain compartmentalized endo- and exoproteases, which are vital for a number of cellular processes, including protein catabolism and precursor activation (Steiner, 2011). The catalytic mechanism is used to further characterize the protease family. Based on their key functional active-site residue(s), proteases are broadly characterized as shown in Table 5.25.1.

Proteolysis is a naturally occurring event within all cells and is necessary to maintain homeostasis (King et al., 1996). Proteases control cellular processes by removing denatured or misfolded proteins, thus eliminating a potentially dangerous buildup of unwanted protein material and simultaneously reducing the requirement for new cellular building blocks through catabolic "recycling." The La protease, a product of the *lon* gene, is responsible for hydrolysis of abnormal proteins in *E. coli* (Chung and Goldberg, 1981). Other roles for proteases include their involvement in apoptosis (Vandenabeele et al., 2005) and a possible role as "signaling scissors," wherein regulated intramembrane proteolysis controls signaling

Table 5.25.1 Classification of Protease Families Based on Catalytic Active Site

Type	Active-site residues	Example
Serine protease	Nucleophilic serine	Subtilisin (EC 3.4.21.62)
Cysteine protease	Nucleophilic cysteine	Papain (EC 3.4.22.2)
Aspartic protease	Two highly conserved aspartate residues	Plasmepsin (EC 3.4.23.39)
Metalloproteinase?	Catalysis involves a metal, often zinc	Adamalysin (EC 3.4.24.46)

in some receptors in the cell membrane (Buckingham, 2003).

In general, proteases are most effective in their native cellular surroundings, where they are often compartmentalized into different subcellular environments. This physical separation reduces nonspecific or nonrequired protease action. Furthermore, protease activity is regulated within these environments (Vanaman and Bradshaw, 1999). However, to access proteins, either native or recombinantly expressed within the cellular environment, these cellular compartments must be disrupted or destroyed. Once these compartments are compromised, proteases can also access proteins from which they are normally physically separated. This is particularly important during protein purification. Careful consideration of the types of proteases present in the cell is required when attempting to reduce proteolysis during protein expression and subsequent purification.

STRATEGIES FOR AVOIDING PROTEOLYSIS

A number of strategies have been described to prevent proteolysis of proteins, both native and recombinant, during their expression. The majority of the examples described below refer to recombinant protein expression in *E. coli*, the most widely used host for protein expression (Samuelson, 2011). Other expression hosts are available, such as yeast strains, insect cells, and mammalian cells (Demain and Vaishnav, 2009). These other expression systems will have their own issues with protease activity, and similar strategies may be employed (see Martensen and Justesen, 2001). These will need to be approached on a case-by-case basis. A helpful review on protein expression system choices has been prepared by an international consortium of researchers (see Structural Genomics Consortium et al., 2008).

Use of Protease-Deficient Cells

Probably the simplest method to avoid proteolysis during expression is to use a commercially available protease-deficient host cell line. *E. coli* mutant cell lines that have been genetically engineered to reduce the effect of, or remove, native *E. coli* proteases are commercially available. For example, the *E. coli* strain BL21 (and its derivatives) is deficient in two proteases encoded by the *lon* (cytoplasmic protease) and *ompT* (periplasmic protease) genes. ?This strain is denoted by the phenotype Δ*lon* or Δ*ompT*, respectively.? The K12 KS1000 strain is lacking the Prc protease, which can degrade proteins expressed in the cytoplasm, while the K12 PR1031 strain is deficient in DnaJ, a chaperone that promotes protein degradation. The CAG597 and CAG629 strains are deficient in stress-induced proteases (see Ryan, 2011).

Optimization of Expression

Another simple but effective method to reduce protease activity is to manipulate the expression conditions to achieve a reduction in the level of misfolded heterologous proteins, which often has the effect of avoiding proteolysis.

Temperature

One of the easiest parameters to alter is incubation temperature. Reduction in temperature results in the slower production of recombinant protein and can result in improved protein folding (Baneyx and Mujacic, 2004; Sörensen and Mortensen, 2005). Temperature is known to play a pivotal role in cellular processing; for example, some proteases can function as chaperones (a "helper" protein) at low temperatures, but act as proteases at elevated temperatures (Leidhold and Voos, 2007). Specialized vectors have been developed for expression of highly sensitive proteins at low temperatures (Mujacic et al., 1999).

Induction conditions

The concentration of the inducing reagent [isopropyl-β-thio-galactoside (IPTG) in the case of commonly used *lac* operator–controlled expression] is another parameter that can be easily altered to achieve a decrease in production of misfolded proteins. With any optimization protocol, only one variable should be altered in any single experiment, and the effect of changing this parameter should be assessed over a feasible range on a small scale. For example, the effect of temperature on recombinant protein production may be examined by varying the incubation temperature from 20°C to 37°C in ~5°C increments, using 10 ml of culture broth. Once one variable has been optimized, keep that variable constant and repeat the optimization process with another variable, e.g., concentration of inducing reagent. The point at which inducer is added may also be significant. Thus, Galloway and co-workers (2003) have reported increased protein yield when the induction of protein expression was initiated in a late-log-phase culture. ?Also, if induction time runs for several hours, then periodically checking protein expression during the induction phase may indicate a window for production of intact protein.?

Use of Fusion Constructs

Misfolded proteins may be targets for proteases if they do not form inclusion bodies (Baneyx and Mujacic, 2004). Fusion of the expressed protein of choice to a protective chaperone may offer significant benefit (Terpe, 2003). The oftentimes larger fusion protein can increase the solubility of the expressed protein of choice and can provide a "handle" for a single-step purification (Cheng and Lee, 2010). Examples of commonly used fusion proteins include maltose binding protein (MBP), N-utilizing substance A (NusA), and glutathione S-transferase (GST). These can all be produced by using commercially available vectors with specialized multiple cloning sites adjacent to the fusion gene to permit simple cloning and co-expression. One drawback to including a fusion protein in an expression and purification strategy is the requirement to remove the fusion protein from the protein of choice after purification. This, ironically, often takes the form of a proteolytic step employing a highly specific, and sometimes expensive, protease. This cleavage step should be completed as efficiently as possible and the protease removed by exhaustive dialysis.

Expressed Protein Targeting

Expressed proteins can be targeted to specific cellular compartments where they are less likely to encounter proteases. In *E. coli*, the typical example is targeting expression to the periplasmic space, which has fewer proteases than the cytoplasm. This is particularly effective for proteins that that require disulfide bonds for activity (Baneyx and Mujacic, 2004). The inclusion of a leader sequence 5′ to the gene of interest, such as the *pelB* leader, is used to direct translocation of the recombinant proteins to the periplasmic space (Barth et al., 2000). This strategy has resulted in many reports of successful translocation of expressed proteins (see Mergulhão et al., 2005 and references within). Because the periplasm contains fewer proteins than the cytoplasm, this strategy allows the protein of choice to be selectively released from the periplasmic envelope by gentle cell lysis (French et al., 1996). For some applications, the recombinant protein may be secreted into the culture medium. In this way, cell lysis is not required to harvest the protein of choice and the cellular proteases are not released (Ni and Chen, 2009). Proteins secreted into the culture supernatant can be collected effectively, as outlined by Caldwell and Lattemann (2004).

Genetic Engineering of Gene Construct

Proteases often cleave proteins at specific amino acid sequences. These residues can be identified by analyzing the gene of the recombinant protein for such cleavage sites. If deletion of these sites does not affect the functionality of the expressed protein, this strategy may be used to prevent proteolysis. As mentioned above in **Use of Fusion Constructs**, some purification protocols require the cleavage of a fusion protein; in these cases, the addition of specific protease recognition sites between the fusion protein and the protein of choice is required. Common examples of these types of recognition motifs include D-D-D-K for the enterokinase protease or E-N-L-Y-F-Q-G for the ?Tobacco Etch Virus? (TEV) protease.

A more radical approach is to randomly mutate the gene of interest to increase the stability of the protein of interest. One method of achieving this is circular permutation; this involves the fusion of the N- and C-terminal ends the production of a new set of termini at a different location within the protein (Luger et al., 1989). Whitehead and co-workers (2009) have used this strategy to

reduce proteolytic cleavage and hence improve the half-life of a molecular chaperone from *Methanocaldococcus jannaschii* expressed in *E. coli*. However, with all such experiments care must be taken to retain protein functionality. Reduced proteolysis, although an important goal, cannot overshadow functionality of the recombinant protein of choice. If this is the case, an alternative strategy should be implemented to reduce proteolysis.

STRATEGIES FOR PREVENTING PROTEOLYSIS

In addition to avoiding proteolysis, it is possible to prevent or minimize the action of proteases by using specific strategies. Some common strategies used for expression in *E. coli* are mentioned elsewhere (see Peti and Page, 2005). The same principles may be extended to other expression systems.

How Do You Know Whether the Expressed Protein has Been Proteolyzed?

It can sometimes be very difficult to determine whether poor expression is due to problems with inclusion body formation, expressed protein toxicity, culture conditions, or proteolysis during cell disruption. On occasion, it may be possible to observe electrophoretic microheterogeneity of an expressed protein band, but this is not always detectable. As a result, measures to reduce proteolysis may have to be taken in the absence of conclusive proof that this is an issue. This matter is further complicated when proteolysis is a consequence of misfolding due to rapid synthesis, i.e., only misfolded protein is hydrolyzed (see Vera et al., 2005). When proteolysis during expression is suspected, consider undertaking some of the more common initial measures outlined above. However, these strategies may not solve the proteolysis issue, and some further strategies aimed at minimizing proteolysis during cell disruption are outlined below. Of course, some of these may have drawbacks in terms of cost or time.

Preventing Proteolysis During Cell Disruption

As mentioned above, cell disruption is a critical period when proteases are liberated from membrane fragments and cellular compartments and begin acting on susceptible recombinant proteins. Cell disruption typically involves resuspending cells expressing a recombinant protein in a lysis buffer and break-ing the cells open using sonication, freeze-thaw cycles, and/or agents such as lysozyme or detergents (e.g., Triton X-100). It is critical that this cell lysis stage is carried out in a way that will minimize protein degradation, as described below.

When proteolysis is an issue during cell disruption, the following strategies may serve to limit proteolytic damage to expressed proteins:

Keep everything cold

Cell lysis and extraction are best carried out at low temperatures. Typically, an extraction buffer is ice cold and lysed cells are kept on ice until centrifugation. The low temperature slows the action of proteases.

Work quickly

For the most part, working quickly will serve to minimize proteolysis. The object is to purify the protein of interest as quickly as possible in order to minimize its contact with a protease. It is important to carry out cell disruption and subsequent chromatography on the same day. This avoids a situation in which the protease is in contact with the expressed protein for a prolonged period. Rapid purification is much easier at a laboratory scale than at a production scale.

Addition of protease inhibitors

A frequently used strategy is to add protease inhibitors to the extraction buffer to inhibit proteases liberated from subcellular compartments. Protease inhibitors are molecules that block the action of proteases either by covalent modification or by a specific interaction (Ryan, 2011). There are a number of commercially available protease inhibitors that may be added at the cell lysis stage either singly or as a cocktail. It is important that these are added as close to the time of cell breakage as possible, as many have short half-lives in solution (see below). Some of these inhibitors act by covalent modification of active-site residues of specific proteins. For example, phenylmethanesulfonyl fluoride (PMSF) reacts with an active-site serine in serine proteases. Some caution is needed in its use since it can modify the protein of interest if it has a susceptible serine residue. It is important to remember that PMSF is unstable in aqueous solution and needs to be added to cell lysis buffers *immediately* prior to use. Table 5.25.2 lists some examples of commonly used protease inhibitors.

Table 5.25.2 Some Examples of Protease Inhibitors Used for the Four Main Classes of Protease[a]

Inhibitor	Concentration in lysis buffer	Stock solution to prepare
Serine protease inhibitor		
AEBSF[b]	0.1-1.0 mM	100 mM in water; store for 1 month at −20°C
PMSF	0.1-1.0 mM	200 mM in isopropanol, freshly prepared. Add to lysis buffer *immediately* prior to use
Leupeptin	10-100 μM	10 mM in water; store frozen for up to 6 months
Cysteine protease inhibitor		
N-ethylmale-imide	Equimolar with protease to be inhibited	10 mg/ml in water, freshly prepared
Antipain	1-100 μM	10 mM in water; store for 1 month at −20°C
Aspartate protease inhibitor		
Pepstatin	1.0 μM	1.0 mM in methanol; store at −20°C
α 2-macro-globulin	Equimolar with protease to be inhibited	Prepare in water; store at −20°C
Metalloproteinase inhibitor		
EDTA	1-10 mM	100 mM in water; store up to 1 year at −20°C
Bestatin	1-10 μM	1.0 mM in water; store up to 1 month at −20°C

[a]Further information can be found in Ryan (2011).
[b]?4-(2-Aminoethyl)benzenesulfonylfluoride.?

Many of these inhibitors have limited specificity while others are specific for more than one class of protease. Trial experiments will be needed to establish which is appropriate for a given situation. A convenient starting point, when proteolysis is suspected, is to use one of the commercially available protease inhibitor cocktail mixes.

It is important to note that the list of inhibitors in Table 5.25.2 is far from exhaustive and that additional inhibitors are commercially available (Ryan, 2011). A database of proteases and their inhibitors has been established that can be a useful source of reference (Rawlings et al., 2012).

Preventing Proteolysis of Proteins During Purification

In general, proteolysis of proteins during purification of recombinant proteins is not a problem if steps have been taken to inactivate proteases at the cell lysis stage. On occasion, however, proteases may be carried over with a purified protein. This may be observed as gradual protein degradation during storage and can be addressed by further purification or by re-application of specific protease inhibitors. The former is always preferable and a variety of chromatographic methods may need to be investigated (see Chapters 8 and 9).

It is important to be sure that loss of activity during purification is not due to causes other than proteolysis. There are a finite number of reasons for loss of activity or function of a protein during purification, ?and these are described below.?

Oxidation

Proteins that require a reduced thiol (on a cysteine residue, for example) for activity or function may become oxidized during purification. Thiols can be maintained in a reduced state by addition of β-mercaptoethanol or DTT (1 to 2 mM).

Loss of cofactor or activating metal ion

Some proteins require a cofactor or a metal ion for proper function. This can be lost during purification. Buffers containing EDTA (used for inhibition of metalloproteinases) are often

a culprit since the EDTA can chelate metal ions. The solution is to exchange into a buffer lacking EDTA but containing the appropriate metal ion or cofactor.

Inactivation by buffer components

Some buffer salts may inactivate certain enzymes. The solution is to exchange into a different buffer.

Inhibition by metal ions

Certain divalent cations may interact with protein thiols to cause inactivation. EDTA is often added at a concentration of 2.0 mM to prevent this inactivation.

All of these patterns of inactivation may be mistaken for proteolysis, thus causing inappropriate action to be taken. Therefore, it is essential to eliminate these causes before considering proteolysis in the absence of specific evidence (see Simpson, 2010 for a review of protein stability).

SPECIAL PROBLEM OF EXPRESSION OF PROTEIN FRAGMENTS

A particular problem may arise when an attempt is made to express a truncated protein or a particular domain of a protein that may not be an issue when expressing the full-length protein. According to some studies, this may be overcome by extending the ends by up to 20 amino acids (see Sörensen and Mortensen, 2005). This issue has been studied in detail by several groups, and systematic approaches to preventing proteolysis by terminal modifications have been reported (Bowie and Sauer, 1989; Structural Genomics Consortium et al., 2008).

SUMMARY

Proteolysis can occur at many stages during the production and purification of a recombinant protein. If protein degradation is suspected at any stage, it is crucial to examine the expression and purification protocols to ensure that true proteolysis has occurred. If proteolysis has been confirmed, alternative methods can be used during expression, cellular disruption, and purification to minimize protein degradation. It may be helpful to identify the type of protease involved in order to design a customized protocol to avoid proteolysis. However, investigation of the type of protease is an empirical process and must be carried out each individual protein. In all cases

it is a better strategy to avoid proteolysis altogether than to have to prevent it after cell lysis and purification.

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Production of Recombinant Proteins

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