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Barry J. Ryan

Gary T. Henehan

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Barry J. Ryan and Gary T. Henehan.

School of Food Science and Environmental Health, Dublin Institute of Technology, Cathal Brugha Street, Dublin 1, Republic of Ireland.

Email: barry.ryan@dit.ie

Abstract

All cells contain proteases which hydrolyse the peptide bonds between amino acids in a protein backbone. Typically, proteases are prevented from non-specific proteolysis by regulation and by their physical separation into different sub-cellular compartments; however, this segregation is not retained during cell lysis, which is the initial step in any protein isolation procedure. Prevention of proteolysis during protein purification often takes the form of a two-pronged approach; firstly inhibition of proteolysis *in situ*, followed by the early separation of the protease from the protein of interest via chromatographical purification. Protease inhibitors are routinely used to limit the effect of the proteases before they are physically separated from the protein of interest via column chromatography. Here, commonly used approaches to reducing or avoiding proteolysis during protein purification and subsequent chromatography are reviewed.

Key Words

Protease, Proteolysis, Protease Inhibitor Buffer, Protein Purification.

1. Introduction

Protein stability can be defined as *"the persistence of molecular integrity or biological function despite adverse influences or conditions, such as heat or other deleterious conditions"* **(*1*)**. One of the key deleterious conditions during protein chromatography is the presence of proteolytic enzymes, referred to as proteases. Proteolysis is the directed degradation of proteins by specific proteases and occurs ubiquitously in nature as homeostatic levels of proteins in cells are governed by a fine balance between their rates of synthesis and their rates of degradation (*see* **Fig. 1**). Proteases have been referred to as "*Nature's Swiss Army knife*" due to their diverse roles in protein cleavage **(*2*)**.

Proteases are employed by all living cells to maintain a particular rate of protein turnover by continuous degradation and synthesis of proteins. Catabolism of proteins provides a ready pool of amino acids that can be reused as precursors for protein synthesis. Intracellular proteases participate in executing correct protein turnover for the cell; for example, in *E. coli*, the ATP-dependent protease La, the *lon* gene product, is responsible for hydrolysis of abnormal proteins **(*3*)**. The turnover of intracellular proteins in eukaryotes is also affected by a pathway involving ATP-dependent proteases **(*4*)**.

Issues with proteases are less acute when purification is from a recombinant host such as *E. coli* since such hosts have been engineered to minimize proteolysis. Nonetheless, issues can arise that may be avoided by judicious choice of expression host. Proteins purified directly from "native" tissues are a different matter and present a far greater challenge. In the latter case the lysis of subcellular organelles may cause the release of a number of ill defined, damaging proteases. In this review the term "protein of interest" will be used to denote the target protein, recombinant or otherwise, to be purified.

[INSERT FIGURE 1 ABOUT HERE]

Originally, proteases were thought to be involved solely in the degradation of unwanted proteins, but they are now known to take part in a wide range of important signalling and physiological processes such as angiogenesis, apoptosis and blood clotting (***5***). The ability of many proteases to digest proteins at known cleavage sites has given rise to a host of biotechnological applications for these enzymes (***6***). Research in this area now employs bioinformatics (***7***) and systems biology (***8***) amongst other tools to investigate the complex interlinked proteome. Based on this research some proteases are recognised as biomarkers for disease states and have become the target of therapeutic intervention in an attempt to modulate a variety of signalling pathways (***9***).

2. Protease Classification

Proteases belong to the hydrolase class of enzyme (Enzyme Classification 3.4), which catalyse the hydrolysis of various bonds with the participation of a water molecule. The proteolytic process involves the cleavage of peptide bonds that link amino acids together in the polypeptide chain. Proteases are defined as either exopeptidases or endopeptidases depending on their site of action (*see* **Table 1**). Proteases are also categorised into four major groups according to their catalytic active site and mode of action (*see* **Table 2**). Another classification divides proteases on the basis of their pH optimum. Thus, acid proteases are optimally active at acid pH values: these were originally identified in the mammalian stomach as components of digestive juices (*e.g.*

Chymotrypsin). Neutral proteases and alkaline proteases are optimally active at neutral and alkaline pH values respectively.

[INSERT TABLE 1 AND 2 HERE]

3. Proteolysis

3.1 Proteolysis during protein purification: prevention is better than cure

Proteases are essential components in all life forms and in normal circumstances proteases are typically packaged into specialised organelles to minimise the chance of unwanted proteolytic activity. Within these organelles there are specific regulators associated with each protease, controlling the action of the protease. Prior to any chromatography step it is necessary to lyse or disrupt cells to liberate the protein of interest in soluble form. It is at this stage that a protein is most vulnerable to proteolysis. When cells are disrupted prior to chromatography-based purification, proteases that are normally located in a different sub-cellular compartments are separated from their regulator molecules and exposed to the protein of interest, thus increasing the probability of undesired proteolysis **(*10*)**. Realistically, it is impossible to remove all proteases present in a chromatography sample preparation, however, careful selection of host cell (if protein of choice is recombinantly expressed) or cell type (if the protein of choice is native) in conjunction with specific sample preparation protocols can reduce unwanted proteolysis during purification **(*11*)**. Approaches to reduce proteolysis during heterologous protein expression and native protein extraction for chromatography purposes will be discussed here by way of examples.

3.2 Reducing Proteolysis during heterologous protein expression

During the production of recombinant proteins, the protein of interest may be exposed to a host protease to which it is particularly susceptible. There are many design strategies that can be enacted to reduce such a potential proteolytic effect. The gram-negative bacterium *E. coli* is the most widely used host for heterologous protein expression in both research laboratories and industry. The approaches below refer to expression in this host, but will apply to other organisms in many cases.

3.2.1 *Use of alternative expression strains:*

Simply altering the host strain may reduce proteolysis of recombinant proteins. There are many commercially available protease deficient strains for heterologous protein expression; for example *E. coli* BL21, is deficient in two proteases encoded by the *lon* (cytoplasmic protease) and *ompT* (periplasmic protease) genes, while other strains lack *Prc* and *DnaJ* protease genes (***12***, *see* **Table 3**).

3.2.2 *Targeting of expressed protein*:

Proteins may be expressed in a subcellular compartment where they are less likely to encounter a protease. Targeting to the periplasmic space of *E. coli*, or even extra-cellularly, by use of co-expressed signaling sequences may avoid degradation by cytoplasmic proteases during expression. This approach, combined with selective cell lysis, will reduce the likelihood of the expressed protein coming in contact with cellular proteases.

3.2.3 *Reduce protein misfolding*:

Protein degradation by proteolysis occurs naturally when mis-folded proteins are produced in the cell. Misfolded proteins are commonly produced during heterologous protein expression and can occur due to a difference in codon usage between *E. coli* and the expressed protein's native environment. The codon bias may be reduced by using specific cell lines (e.g. *Oragami*™ or *pRARE*™ from Novagen), lowering growth temperature (*see* ***13*** for an in-depth review) or by altering the growth medium (e.g. polyol inclusion (***14***)).

3.2.4 *Use of fusion proteins:*

Another approach to protect recombinant proteins from unwanted proteolysis is to fuse them to a protein tag. These tags can improve protein folding and solubility and also act as a convenient handle for purification. Examples here include proteins fused to Glutathione-S-Transferase (GST) or Maltose Binding Protein (MBP). Vectors incorporating such tags are commercially available; GST (pGEX plasmid system from *GE Healthcare*) and MBP (pMAL plasmid system from *New England Biolabs*).

3.2.5 Alternative expression hosts:

If the above approaches fail, it may be necessary to consider expression in another heterologous host (e.g. another prokaryote, yeast or mammalian cell line). A number of commercially available expression systems have been developed in recent years (***15***, ***16*** and ***17***).

3.3 Proteolysis reduction during native protein purification

The purification of proteins from cells (plant or animal) other than specialized prokaryotic expression hosts can be more problematic. In the case of eukaryotic cells, for example, cell lysis may cause the release of lysosomal proteases that may attack a protein of interest. In this case a number of basic procedures to minimize protease activity are required.

3.3.1 Source of native protein:

Native protein sources, such as mammalian tissues, often exhibit differing protease levels (e.g. liver and kidney samples contain a much higher concentration of proteolytic enzymes than skeletal or cardiac muscle, ***18*)**. Careful selection of protein source may sufficiently reduce protease activity during extraction and purification.

3.3.2 Low temperature lysis:

Cell lysis is normally carried out on ice to keep degradative processes to a minimum. The extract buffer should also be chilled to 4^{o}C before use.

3.3.3 Work quickly: It is important to prepare a clarified cell extract for purification as quickly as possible to minimize contact between the protease and the protein of interest. A clarified extract is normally achieved by centrifugation using a refrigerated centrifuge. It is important to proceed to the first purification step as quickly as possible after cell lysis and clarification. The initial purification step should be designed to separate the protein of interest from proteases.

3.3.4 Control of pH:

Typically cell lysis is carried out at neutral or slightly alkaline pH. This will minimize the activity of acid proteases; however, it will not affect neutral or alkaline proteases.

3.3.5 Lysis buffer additives:

A range of compounds may be added, sequentially or simultaneously, to the lysis buffer to reduce protease action. Salts may be added to alter the osmotic concentration of buffer, glycerol (5-15% v/v) and sucrose (2.5 m*M*) may be added to stabilize proteins. In addition to these compounds there are a range of specific protease inhibitors that may be added to lysis buffers to stabilize proteins (*see* **Table 4**). Many of these agents are commercially available as inhibitor cocktails or they can be made in-house (*see* **Table 5** and **Section 4.3**).

3.3.6 Alternative approaches:

In some cases it may be possible to heat shock an extract to temperatures up to 70^0C or more; however, this will *only* work if the protein of interest is heat stable. The heat shock inactivates degradative enzymes, such as proteases, while maintaining the activity of the protein of interest. Another approach is to use salting-out to precipitate and stabilize the protein of interest. In this process ammonium sulphate is added (slowly, with stirring) up to 70% w/v saturation to render proteins insoluble. The precipitated proteins may be collected by centrifugation. Many proteins are surprisingly stable as precipitates and can be stored in this form for extended periods before being resolubilised by a simple dialysis step.

Once the source of the protein of interest has been optimized, a commonly used approach toward prevention of further unwanted proteolysis during protein isolation and purification is to include protease inhibitors during sample preparation, purification, and characterization.

[INSERT TABLE 3 ABOUT HERE]

4. Protease Inhibition

4.1. Protease Inhibitor Selection and Preparation

Proteolysis avoidance, or reduction, is better than dealing with a protease after it has begun to act. However, if proteolysis is unavoidable, understanding the protease you are dealing with will help in choosing alternative protein isolation and purification strategies or, in the worst case, selecting a suitable inhibitor. One should also consider whether the protease activity is a problem all of the time, or only during certain conditions (e.g. stressful induction, isolation from cancer cell lines, *see **19***). Judicious inhibitor choice will depend on the correct empirical identification of the protease involved.

The identity of a protease(s) can be determined in several ways, however, the simplest method is to incubate the sample of choice with a single inhibitor from the group of inhibitors (Serine, Cysteine, Thiol, Metallo- etc.) listed in **Table 2**. The degree of proteolysis can be simply identified from Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS PAGE) analysis of the protein sample post-inhibitor incubation; increased protein band smearing on the gel or a change in expected protein size will indicate potential proteolysis. Proteolysis inhibition, indicated by a maintenance of correct protein size with no protein band smearing after a given

incubation period with inhibitor, will permit the identification of a suitable inhibitor group for the sample preparation. There are alternative strategies for the specific identification of proteases including fluorescence labelling (***20***), substrate tagging (***21***), zymography (***22***) and activity based probes (***23***); however, these techniques are generally more expensive and labour intensive. The type of proteolysis encountered in a given tissue sample can be divided into two broad categories:

Minor hydrolysis: An exopeptidase that cleaves off one, or more, terminal amino acids may cause little disruption to the integrity or function of a protein. This degree of degradation may go unnoticed as catalytic activity, for example, may be unchanged. In some cases, significant cleavage of an enzyme by endopeptidases may occur without loss of activity or function. Such proteolysis may only be detected as electrophoretic heterogeneity or by Mass Spectroscopy. A consequence of this hydrolysis where protein activity or function is partially lost may be extremely difficult to detect.

Catastrophic hydrolysis: In this case hydrolysis is such as to render a protein devoid of activity or function. The protein may not be detectable by traditional techniques such as activity assay or using antibody probes. In this case it may be useful to monitor lysates for protease activity using an appropriate screening assay.

Once the type of protease has been identified, individual inhibitors can be chosen from **Table 4** or a typical *general-use* protease inhibitor mix can be prepared immediately before use from the stock concentrations outlined in **Table 5**. Protease inhibitor solutions must be correctly stored after they have been prepared. Aliquot a stock solution of inhibitor and store it at the correct

temperature (*see* **Table 4**) to maintain the properties of the inhibitor. Make small, single use aliquots to reduce the risk of stock contamination. Ensure that the protease inhibitor/inhibitor mix is combined with the cell sample *immediately prior* to cell disruption. If the individual protease inhibitor/inhibitor mix is to be prepared fresh then it must be used within one hour of preparation.

[INSERT TABLE FOUR ABOUT HERE]

[INSERT TABLE FIVE ABOUT HERE]

It should be noted that the generic protease inhibitor cocktail outlined here is not guaranteed to work in all circumstances. The success of any mix will depend on the correct empirical identification of the protease involved.

4.2 Commercially available Universal Protease Inhibitor Mixes.

There are several types of commercially available "Universal Protease Inhibitors" that may also be used (e.g. *Complete Protease Inhibitor Cocktail Tablets*, Roche Applied Science). Additionally, many companies offer inhibitor panels, such as the *Protease Inhibitor Panel* (Sigma Aldrich), which is a cost-effective method for personalized protease cocktail inhibitor generation **(*24*)**.

4.3 Supplementary Protease Inhibitor Components.

Additional Inhibitors

If a particular protease is thought to be dominant within a sample preparation, the cocktail mix may be supplemented with additional specific protease inhibitors **(*25- 33*)**. Commonly used specific individual protease inhibitor components are outlined in **Table 6**.

[INSERT TABLE SIX ABOUT HERE]

Phosphatase inhibitors may also be required since many enzymes are activated by phosphorylation: hence dephosphorylation must be inhibited if enzyme activity is to be maintained. Again, an empirical approach is required to identify if a phosphatase inhibitor is required (*see* **Section 4.1** and **Table 7**). Protein phosphatases can be divided into two main groups: protein tyrosine phosphatases and protein serine/threonine phosphatases, which remove phosphate from proteins (or peptides) containing phosphotyrosine or phosphoserine/phosphothreonine respectively (***34***). Inhibitors commonly used include: p-Bromotetramisole, Cantharidin**,** Microcystin LR (Ser/Thr Protein Phosphatases and Alkaline Phosphatase L-Isozymes) and Imidazole, Sodium molybdate, Sodium orthovanadate, Sodium tartrate (Tyr Protein Phosphatases and Acid and Alkaline Phosphatases, *see* **Table 7**). There are also a number of commercially available Phosphatase Inhibitor Mastermixes (e.g. PhosphataseArrest™ Phosphatase Inhibitor Cocktail, Geno Technologies Ltd.). These are often supplied in convenient, ready-to-use 100X solutions that are simply added to the protein extraction buffer or individual samples. These mixes can be sourced as either broad spectrum phosphatase inhibitor cocktails or as phosphatase inhibitors for targeting particular set of phosphatases.

[INSERT TABLE SEVEN ABOUT HERE]

4.4 Supplementary Chemical Compounds including Enzymes.

The addition of supplementary chemical components to disrupt protease activity should be carefully assessed on a small scale since such components may alter the function/stability of the protein of interest (*see* **Table 8**). Moreover, additional protease inhibitors should be introduced to the sample with caution since protein modifications, such as alteration of protein charge, may occur. These alterations may interfere with further protein characterisation studies. For example, 2-mercaptoethanol will reduce the cysteine proteases, but may also unfold target proteins containing disulphide bridges. Ethylenediamine-tetraacetic acid (EDTA) is included in many protease inhibitor buffers because metal ions are frequently involved in proteolysis, thus their removal will impede proteolysis. However, if one is purifying poly-Histidine tagged proteins or metalloproteins, then the chelating effect of EDTA will dramatically alter purification yields, and the EDTA should be removed by dialysis or a buffer exchange resin prior to chromatography. Inclusion of 2 *M* thiourea may also prevent proteolysis: Castellanos-Serra and Paz-Lago **(*35*)** noted the protease inhibitory effects of its addition in conjunction with its efficiency in solubilizing proteins. DN*ase* (100 U/mL), although not itself a protease inhibitor, can be included in the cell lysis buffer as this will serve to reduce the viscosity of the crude lysate. The reaction is allowed to proceed for 10 min at 4°C in the presence of 10 m*M* $MgCl_2$.

[INSERT TABLE EIGHT ABOUT HERE]

4.5 Protease Inhibition During Chromatography.

The introduction of contaminating proteases from your own skin, non-sterile water etc. can be avoided by sterilising all plasticware and by wearing appropriate personal protective equipment. All buffers should be filter sterilised (0.2 µm) into autoclaved bottles (sterile filtering will not remove contaminating proteases, but will remove any protease secreting microorganisms). Additionally, sterile filtration of the protein eluate, once purification is complete, is recommended.

Cell disruption, as with all other parts of the purification procedure, should take place at 2-8^{o}C. This temperature will not only reduce the activity of proteases, but will also aid in stabilizing the target protein (reduction in thermal denaturation). Kulakowska-Bodzon and co-workers **(*36*)** provide an excellent review on protein preparation from various cell types for proteomic work. In general, all buffers and materials should be pre-chilled to 2-8^{o}C. Rapid purification at this lower temperature will reduce the risk of unwanted proteolysis. It is advisable not to store such samples at 2-8^{o}C for more than one day between purification steps, rather store them at -20°C.

Gel filtration (size exclusion chromatography) is often used as the final step in protein purification where it can be used to desalt and buffer exchange the protein (thus eliminating the need for dialysis). Contaminating proteases can also be separated from the protein of choice if there is significant separation between elution peaks for the protease and the protein of choice. This is the case only where there is a considerable difference between the size of the protease and the size of the protein of interest. If a multi-step purification strategy is being used, try to carry out the purification that delivers the best separation between protease and protein of

interest at the beginning. This, however, may not always be feasible, as other factors must be considered in designing a purification strategy (*e.g.* physio-chemical properties of the target protein, cost and time).

5. Proteases in chromatography

5.1 Use of proteases during chromatography

Some purification protocols require the addition of specific proteases. Common examples here include the use of *enterokinase* (recognition site D-D-D-K) or *TEV protease* (recognition site E-N-L-Y-F-Q-G) to remove polypeptide and protein purification tags from recombinant proteins. More recently, designed and non-specific proteolysis during preparative chromatography has been used to assist in glycoprotein characterisation (***37*** and ***38***), lipid protein purification (***39***) and antibody profiling (***40***). In all cases, it is critical to ensure that any protease inhibitor containing buffer is exchanged, by dialysis or a suitable buffer exchange resin, prior to the addition of the desired protease.

5.2 Post-Chromatographic Analysis.

Protease inhibition can be either reversible or irreversible. The majority of serine and cysteine protease inhibitors are irreversible, whereas the aspartic and metalloprotease inhibitors are reversible. Even when the inhibitors are added at an early stage, they may be lost during

purification and subsequent handling steps, resulting in proteolysis post-chromatography. The further re-addition of protease inhibitors may therefore be necessary as purification progresses.

Even with increased numbers of purification steps, very few protocols will remove all proteases from a sample preparation however one can hope to achieve an adequate reduction in the level of these contaminants. Each purification protocol will have a unique definition of "*adequate protease reduction*" based on a number of variables including the activity of the remaining proteases, further downstream applications of the protein of choice and the cost of further protease removal. Additional purification steps often result in a reduced final yield, as such the trade-off between contaminant reduction and yield must be optimised.

An apparently pure protein that gives a single band on a Coomassie-stained SDS-PAGE gel should be re-analysed over time to ensure minimal protease activity exists in the purified sample. This may be carried out by simply storing an aliquot of the purified protein solution at room temperature and analysing samples of this by SDS-PAGE at regular intervals. If the protein is being degraded (indicated by a smear or a reduced size of the protein of choice), protease contamination is present and an additional purification step (or supplemental inhibitor addition) is required.

Care must be taken to rule out the possible loss of enzyme activity due to other destabilizing factors during protein purification. These other factors include, but are not limited to, thermal denaturation, oxidative damage and column matrix adherence. Thermal denaturation of proteins is the decreased stability of a protein caused by extremes of temperature. Thermal denaturation

can be reduced if the purification procedure is carried out at 2-8 ^{o}C. All buffers and chromatography columns/resins should be pre-chilled to 2-8 ^{o}C and the purified protein stored at the correct temperature.

Oxidative damage to proteins can be divided into a number of categories, however improper disulphide formation is the most pertinent here. Thiol oxidation may be crucial for correct protein folding. The formation of incorrect intra- or intermolecular disulfides is a detrimental process that can often result in loss of activity and/or aggregation. Oxidative damage can be avoided by not exposing the protein of interest to thiol reducing compounds (e.g. β-mercaptoethanol) during purification thus maintaining the correct folded state of the protein.

Column matrix adherence is caused by the binding of the protein of interest to the purification column support material by virtue of its physicochemical properties (e.g. surface charge or hydrophobicity). Non-specific protein adherence can cause sheer stress damage to the protein during purification, however this can be circumvented by careful selection of the purification column (type/grade of glass or plastic) and purification resin.

6. Conclusion

The presence of proteolytic enzymes can result in target protein degradation during protein chromatography. Careful selection of source organism/tissue, along with judicious use of protease inhibitors, can reduce these degrading effects. Commonly used inhibitors are listed here in tabular format (*see* **Tables 4** and **5**), along with supplemental compounds (*see* **Tables 6** and **7**) for easy selection. Protease inhibitors can be added individually or as part of a mix, however, optimal inhibitor selection is an empirical process.

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Table 1: Broad classification of proteases based on their site of action along a protein chain.

Peptidase Type	**Mode of Action**	**Common Examples**
Exopeptidase	Hydrolyse the terminal amino acids from the protein chain. They may act on the amino, or carboxyl, terminal of the peptide chain.	Aminopeptidases and Carboxypeptidase
Endopeptidase	Hydrolyse internal peptide bonds of a protein, common examples here include trypsin, chymotrypsin, pepsin, and papain	Trypsin, Chymotrypsin, Pepsin, and Papain

Table 2: Broad classification of proteases based on their active site amino acid or metal ion (***41***).

Protease Type	**Active Site Amino Acid or Metal Ion**	**Common Examples**
Serine Protease	Serine	Subtilisin (EC 3.4.21.62, an endopeptidase sourced from *Bacillus subtilis*, ***(42)***)
Cysteine (thiol) Protease	Nucleophilic cysteine thiol	Papain (EC 3.4.22.2, an endopeptidase sourced from *Carica papaya*, ***(43)***)
Aspartate Protease	Aspartic Acid (in general, two highly-conserved aspartate residues)	Plasmepsin (EC 3.4.23.39, an endopeptidase produced by the *Plasmodium* parasite, ***(44)***)
Glutamate Protease	Glutamate and Glutamine dyad.	Glutamate carboxypeptidase 2 (EC 3.4.17.21, an exopeptidase sourced from *Homo sapiens*, ***(45)***)
Threonine Protease	Threonine (commonly activated by a Histidine)	TSP50 peptidase, an endopeptidase sourced from *Homo sapiens*, ***(46)***).
Asparagine Protease	Asparagine (commonly as a dyad with an acidic residue)	MeTr peptidase, an endopeptidase sourced from *Homo sapiens*, ***(47)***).
Metalloproteases	Catalysis requires an active site metal ion (e.g. zinc or cobalt)	Adamalysin (EC 3.4.24.46, an endopeptidase from the rattlesnake *Crotalus adamanteus*, ***(48)***).

Table 3: Some commercially available protease-deficient *E. coli* strains that are used to express recombinant proteins.

Strain Name	**Protease Deficiency**	**Supplier**
UT5600	Deficient in *OmpT* (an outer membrane protease that cleaves between sequential basic amino acids).	New England Biolabs Inc.
CAG626	Deficient in Lon (a protease that degrades abnormal/misfolded proteins).	New England Biolabs Inc.
CAG597	Stress-induced proteases at high temperature.	New England Biolabs Inc.
CAG629	Stress-induced proteases at high temperature and Lon protease.	New England Biolabs Inc.
PR1031	Deficient in DnaJ –a chaperone that can promote protein degradation.	New England Biolabs Inc.
KS1000	Deficient in Prc (Tsp), a periplasmic protease.	New England Biolabs Inc.
Rosetta	Deficient in Lon and OmpT.	Novagen
Rosetta-gami B	Deficient in Lon and OmpT.	Novagen
Origami B	Deficient in Lon and OmpT.	Novagen
BL21 Star(DE3)pLysS	Deficient in Lon and OmpT.	Invitrogen
BL21 Star(DE3)	Deficient in Lon and OmpT.	Invitrogen
BL21-AI	Deficient in Lon and OmpT.	Invitrogen

Table 4: Protease-Inhibitors: Stock Solutions and Storage Conditions.

Inhibitor Activity	Inhibitor	Solvent	Molarity	Storage
Serine	PMSF[1]	dry methanol or propanol	200 m*M*	-20ºC
Serine	3,4-DCL	dimethylsulfoxide	10 m*M*	-20ºC
Serine	Benzamidine	water	100 m*M*	-20ºC
Cysteine	Iodoacetic acid	water	200 m*M*	Prepare fresh
Cysteine	E64-c	water	5 m*M*	-20ºC
Thiol (serine & cysteine)	Leupeptin	water	10 m*M*	-20ºC
Metallo	1,10 Phenanthroline	methanol	100 m*M*	RT[3] or 4ºC
Metallo	EDTA[2]	water	0.5 *M*	RT[3] or 4ºC
Acid Proteases	Pepstatin	DMSO	10 m*M*	-20ºC
Aminopeptidase	Bestatin	water	5 m*M*	-20ºC
Threonine	Leupeptin	water	10 m*M*	-20ºC

[1] PMSF is toxic. Weigh this compound in a fume hood, and wear appropriate personal protective equipment.

[2] Does not inhibit pancreatic elastase.

[3] RT - Room Temperature.

Table 5: General protease inhibitor mix

Stock Inhibitor	**Volume (µL)**
PMSF (100 m*M*) or 3,4-DCI (10 m*M*) or Benzamidine (5 m*M*)	200
Iodoacetate (200 m*M*) or E64-c (5 m*M*)	200
1,10 phenanthroline (100 m*M*) or EDTA (500 m*M*) or Leupeptin (10 m*M*)	100
Pepstatin (10 m*M*)	100
Double Distilled Water	400
Final Volume	1,000

Table 6. Additional inhibitors that can be used to supplement protease inhibitor mixes.

Inhibitor	Solvent	Molarity	Storage
Serine Protease Inhibitors			
Aprotinin (Does not inhibit thrombin or factor Xa)	water	300 m*M*	-20ºC (at pH 7)
Chymostatin (Inhibits chymotrypsin-like serine proteases such as chymase cathepsins A,B,D and G. Also inhibits some cysteine proteases such as papain)	DMSO	10 m*M*	-20ºC
Antithrombin III (Inhibits thrombin, kallikreins, plasmin, trypsin and factors Ixa, Xa, and Xia)	water	10 Units/mL	-20ºC (at pH 7)
TLCK (Inhibits chymotrypsin-like serine proteases)	1 m*M* HCl	100 µ*M*	Prepare fresh
TPCK (Inhibits chymotrypsin-like serine proteases)	Ethanol	10 m*M*	4ºC
DIFP (Highly toxic cholinesterase inhibitor. Broad spectrum serine protease inhibitor. Hydrolyzes rapidly in aqueous solutions)	anhydrous isopropanol	200 m*M*	-20ºC
Antipain (Inhibits serine proteases such as plasmin, thrombin and trypsin. Also inhibits some cysteine proteases such as calpain and papain)	water	10 m*M*	-20ºC
***α*2-Macroglobulin** (Broad spectrum protease inhibitor)	water	100 m*M*	-20ºC
Cysteine Protease Inhibitors			
N-Ethylmaleimide	water	100 m*M*	Prepare fresh
Metalloprotease Inhibitors			
Phosphoramidon (Strong inhibitor of metalloendoproteases, thermolysin and elastases, but a week inhibitor of collagenase)	water	1 m*M*	-20ºC

Table 7: Commonly used phosphatase inhibitors.

Name	Typical Working Molarity Range	Stock Molarity	Typical Inhibitory Targets.
p-Bromotetramisole	0.1 – 1.5 m*M*	100 m*M*	Alkaline Phosphatases (***49***, ***50***)
Cantharidin	20 – 250 μ*M*	2.5 m*M*	Protein Phosphatase 2-A (***49***, ***51***)
Microcystin LR	20 – 250 n*M*	2.5 μ*M*	Protein Phosphatase 1 and 2-A (***49***, **52**)
Imidazole	50 – 200 m*M*	1 *M*	Alkaline Phosphatases (***53, 54***)
Sodium molybdate	50 – 125 m*M*	1 *M*	Acid phosphatases and Phosphoprotein Phosphatases (***51, 54***)
Sodium orthovanadate	50 – 100 m*M*	1 *M*	ATPase inhibition, Protein Tyrosine Phosphatases, Phosphate-transferring enzymes. (***54, 55***)
Sodium tartrate	50 – 100 m*M*	1 *M*	Acid Phosphatases (***52, 54***).

Table 8: Supplemental chemical/enzyme additions to protease inhibitor buffer **(*56*)**.

Item and typical working concentration	Advantages	Disadvantages	Uses / Typical Protease Targets
2-mercaptoethanol (1 m*M*)	Reduction cysteine protease activity.	Unfolding of target proteins containing disulphide bridges	Cysteine Proteases
Dithiothreitol (2 m*M*)	Reduction cysteine proteinase activity. Low odour.	Unfolding of target proteins containing disulphide bridges	Cysteine Proteases
EDTA (5 m*M*)	Removal of metal ions involved in proteolysis impeding proteolysis	The chelating effect of EDTA will affect the structure of metalloproteins and dramatically reduce the purification of poly-Histidine tagged proteins.	Non-His tagged protein targets or non-metalloprotein targets.
Thiourea (2 *M*)	Proteolysis inhibitory effects, in conjunction with improved protein solubilisation.	Thiourea is considered a possible human carcinogen and mutagen.	General purpose protease inhibitor.
Detergents (e.g. SDS or deoxycholate; 2 %v/v)	Useful in solubilising membrane proteins	May activate some proteases	Serine proteases
Sucrose(2.5 m*M*) /Glycerol (5-15 %v/v)	Stabilises proteins	May need to be removed by dialysis	General stabilization of lysosomal membranes to prevent protease leakage
DN*ase* (100 U/mL)	Reduction in the crude lysate viscosity.	Requires further incubation step of 10 min at 4°C in the presence of 10 m*M* $MgCl_2$.	Can be included in the cell lysis buffer for optimal efficiency.

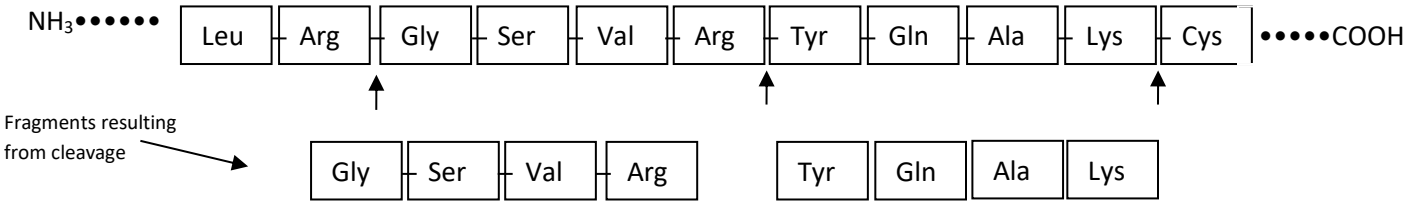

Figure 1: The schematic depicts a peptide chain fragment, indicating where the chain will be cleaved by trypsin. Trypsin cleaves at the carboxyl side of Arginine (Arg) and Lysine (Lys) amino acids. The cleavage points are indicated by arrows. The amino acid fragments resulting from the cleavage are indicated.