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Avoiding Proteolysis During Chromatography.

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Abstract

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

Key Words

Protease, Proteolysis, Proteinase 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 substances, often referred to as proteases. Proteolysis is the directed degradation of proteins by specific proteases. Proteases have been referred to as "*Nature's Swiss Army knife*" due to their diverse applications in protein cleavage **(*2*)**. 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 hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain. Proteases are defined as either exopeptidases (detach the terminal amino acids from the protein chain, examples include aminopeptidases and carboxypeptidase), or endopeptidases (which target internal peptide bonds of a protein, common examples here include trypsin, chymotrypsin, pepsin, and papain) **(*3*)**. Proteases are also divided into four major groups according to their catalytic active site and mode of action: serine proteinases, cysteine (thiol) proteinases, aspartic proteinases, and metalloproteinases **(*4*)**. Serine proteases, as the name suggests, have a serine residue as part of its catalytic site. Subtilisin (EC 3.4.21.62, an endopeptidase sourced from *Bacillus subtilis*) is one of the most common serine proteases examples cited **(*5*)**. Cysteine proteases have a nucleophilic cysteine thiol as part of their active site. Papain (EC 3.4.22.2, an endopeptidase sourced from *Carica papaya*) is a frequently cited example of a cysteine protease **(*6*)**. Aspartic proteinases use an aspartate residue for catalysis and, in general, they have two highly-conserved aspartate residues in the active site and are optimally active at acidic pH. Plasmepsin (EC 3.4.23.39, an endopeptidase produced by the *Plasmodium* parasite) is an example of an aspartic proteinase **(7)**.

Metalloproteinases contain a catalytic mechanism involving a metal; most contain zinc, however, cobalt centres are also observed. Adamalysin (EC 3.4.24.46, an endopeptidase from the rattlesnake *Crotalus adamanteus*) is an example of a metalloproteinase **(*8*)**.

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: in *E. coli*, the ATP-dependent protease La, the *lon* gene product, is responsible for hydrolysis of abnormal proteins **(*9*)**. The turnover of intracellular proteins in eukaryotes is also affected by a pathway involving ATP-dependent proteases **(*10*)**.

As such, proteases are essential components in all life forms and in normal circumstances proteases are typically packaged into specialised organelles to minimise the chance of non-specific proteolytic activity. Within these organelles there are specific regulators associated with each protease, controlling the action of the protease. However, when cells are disrupted for chromatography purification, proteases that are normally located in a different sub-cellular compartment are separated from their regulator molecules and exposed to the protein of interest, thus increasing the probability of undesired proteolysis **(*11*)**. Realistically, it is impossible to remove all proteinases 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 **(*3*)**.

Proteases are ubiquitous and play a crucial role in normal and abnormal physiological conditions in all living things by effecting catalysis throughout many metabolic pathways. However, there is an uneven distribution of proteinases depending on which cell type (bacterial or eukaryotic) or tissue is disrupted. During heterologous protein expression, the recombinant protein of interest may be exposed to a host proteinase to which it is particularly susceptible. Simply altering the host may reduce recombinant proteolysis. Many commercial companies offer 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 (*see* **Table 1**). Additionally in mammalian tissues, liver and kidney samples contain a much higher concentration of proteolytic enzymes compared to skeletal or cardiac muscle **(*12*)**. Once the source of the protein of choice has been optimized, a commonly used approach toward prevention of further unwanted proteolysis during protein isolation is to include proteinase inhibitors during sample preparation, purification, and characterization.

INSERT TABLE ONE ABOUT HERE

1.1 Proteinase Inhibitor Selection and Preparation.

Judicious inhibitor choice will depend on the correct empirical identification of the proteinase involved. Classification of the proteinase(s) can be carried out 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 etc.) listed in **Table 2**. The degree of proteolysis can be 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. Once the protease has been identified, individual inhibitors can be chosen from Table 2 or a typical *general-use* proteinase inhibitor mix can be prepared immediately before use from the stock concentrations outlined in **Table 3**. Proteinase inhibitor solutions must be correctly stored after they have been prepared. Aliquot the stock of inhibitor and store at the correct temperature (*see* **Table 2**) to maintain the properties of the inhibitor. Make small, single use aliquots to reduce the risk of stock contamination. Ensure that the proteinase inhibitor/inhibitor mix is combined with the cell sample prior to cell disruption. If the individual proteinase inhibitor/inhibitor mix is to be prepared fresh then it must be used within one hour of preparation.

INSERT TABLE TWO ABOUT HERE.

INSERT TABLE THREE ABOUT HERE.

It should be noted that the generic proteinase 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 proteinase involved.

1.2 Commercially available Universal Protease Inhibitor Mixes.

There are several types of commercially available "Universal Proteinase 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 proteinase cocktail inhibitor generation **(13)**.

1.3 Supplementary Protease Inhibitor Components.

Additional Inhibitors

If a particular protease is dominant within a sample preparation the cocktail mix may be supplemented with additional specific proteinase inhibitors **(*12,14 - 21*)**. Commonly used specific individual protease inhibitor components are outlined in **Table 4**.

INSERT TABLE FOUR ABOUT HERE.

Phosphatase inhibitors may be required also as 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 1.1** and **Table 5**). 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 (***22***). Inhibitors commonly used here 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 5**). 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 FIVE ABOUT HERE.

1.4 Supplementary Chemical Compounds including Enzymes.

The addition of supplementary chemical components to disrupt proteinase activity first should be carefully assessed on a small scale as often these components will alter the function/stability of the target protein (*see* **Table 6**). Furthermore, additional protease inhibitors should be introduced to the sample with caution as 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 activity of cysteine proteinases, but will also unfold target proteins containing disulphide bridges. EDTA is included in many proteinase inhibitor buffers as 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 from the buffer by dialysis or a buffer exchange resin. Inclusion of 2 *M* thiourea may also prevent proteolyis. Castellanos-Serra and Paz-Lago **(*23*)** noted the proteolysis 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 reduce the overall 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 SIX ABOUT HERE.

1.5 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 sterile filtered (0.2 μm) into autoclaved bottles (sterile filtering will not remove contaminating proteases, but will remove any protease secreting microorganisms). Additionally, sterile filter the protein eluate once purification is complete.

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 proteinases, but will also aid in stabilizing the target protein (reduction in thermal denaturation). Kulakowska-Bodzon and co-workers **(*24*)** 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. Do not store such samples at 2-8^{o}C for more than one day between purification steps, instead store at -20°C.

Gel filtration (size exclusion chromatography, *see* **Chapter 2**) is often used as the final step in protein purification and it can be used to desalt and buffer exchange the protein (therefore no need for dialysis). Contaminating proteinases 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 based on the presumption that there is a considerable difference between the size of the protease and the size of the protein of interest.

1.6 Post Chromatography Analysis.

Protease inhibition can be either reversible or irreversible. The majority of serine and cysteine proteinase inhibitors are irreversible, whereas the aspartic and metalloproteinase 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-purification. The further re-addition of proteinase inhibitors may therefore be required after purification.

Even with increased numbers of purification steps, very few protocols will remove all contaminants from a sample preparation however one can 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. Increased purification steps often result in a reduced final yield, as such the trade-off between contaminant reduction and yield must be optimised. A pure protein that gives a single band on a Coomassie-stained SDS-PAGE gel should be re-analysed over time to ensure minimal proteinase activity exists in the 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), proteinase contamination is present and an additional purification step (or supplemental inhibitor addition) is required.

Some purification protocols require the addition of specific proteases such as *enterokinase* (recognition site D-D-D-K) or *TEV protease* (recognition site E-N-L-Y-F-Q-G) to remove polypeptide tags from recombinant proteins. Ensure that any proteinase inhibitor containing buffer is exchanged, by dialysis or a suitable buffer exchange resin, prior to the addition of the desired proteinase (*see also* **Chapter 19**).

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 surface adherence. Thermal denaturation of proteins is the decreased stability of a protein caused by extremes of temperature experienced by the protein of interest. Thermal denaturation can be reduced if the purification procedure is carried out at 2-8^{o}C, all buffers and chromatography columns/resins are pre-chilled to 2-8^{o}C and if the purified protein is stored at the correct temperature post-purification. Oxidative damage to proteins can be divided into a number of sections; however improper disulfide formation is the most pertinent here. Thiol oxidation is crucial for correct protein folding, resulting in a stabilized 3-D protein structure in proteins containing disulphide bridges. The formation of incorrect intra- or intermolecular disulfides is a detrimental process that can often result in loss of activity and/or aggregation. Thiol oxidative damage can be avoided by not exposing the protein of choice to

thiol reducing compounds (e.g. β-mercaptoethanol) during purification, thus maintaining the correct folded state of the protein. Column surface adherence is caused by the attraction of the protein of choice to the surface of the purification column by the protein's intrinsic physico-chemical 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.

2.0 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 3** and **4**), along with supplemental compounds (*see* **Tables 5** and **6**) 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: 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 2: Protease-Inhibitors: Stock Solutions and Storage Conditions.

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

[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 3: General proteinase 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 4. 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*	-20°C (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*	-20°C
Antithrombin III (Inhibits thrombin, kallikreins, plasmin, trypsin and factors Ixa, Xa, and Xia)	water	10 Units/mL	-20°C (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*	4°C
DIFP (Highly toxic cholinesterase inhibitor. Broad spectrum serine protease inhibitor. Hydrolyzes rapidly in aqueous solutions)	anhydrous isopropanol	200 m*M*	-20°C
Antipain (Inhibits serine proteases such as plasmin, thrombin and trypsin. Also inhibits some cysteine proteases such as calpain and papain)	water	10 m*M*	-20°C
α2-Macroglobulin (Broad spectrum protease inhibitor)	water	100 m*M*	-20°C
Cysteine Protease Inhibitors			
N-Ethylmaleimide	water	100 m*M*	Prepare fresh
Metalloproteinase Inhibitors			
Phosphoramidon (Strong inhibitor of metalloendoproteinases, thermolysin and elastases, but a week inhibitor of collagenase)	water	1 m*M*	-20°C

Table 5: 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 (***25***, ***26***)
Cantharidin	20 – 250 μ*M*	2.5 m*M*	Protein Phosphatase 2-A (***25***, ***27***)
Microcystin LR	20 – 250 n*M*	2.5 μ*M*	Protein Phosphatase 1 and 2-A (***25***, **28**)
Imidazole	50 – 200 m*M*	1 *M*	Alkaline Phosphatases (***29, 30***)
Sodium molybdate	50 – 125 m*M*	1 *M*	Acid phosphatases and Phosphoprotein Phosphatases (***27, 30***)
Sodium orthovanadate	50 – 100 m*M*	1 *M*	ATPase inhibition, Protein Tyrosine Phosphatases, Phosphate-transferring enzymes. (***30, 31***)
Sodium tartrate	50 – 100 m*M*	1 *M*	Acid Phosphatases (***28, 30***).

Table 6: Supplemental chemical/enzyme additions to protease inhibitor buffer **(*32*)**.

Item and typical working concentration	Advantages	Disadvantages	Uses / Typical Protease Targets
2-mercaptoethanol (1 m*M*)	Reduction cysteine proteinase activity.	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.
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.