Removal of N-Terminal Blocking Groups from Proteins

Two enzymatic methods are commonly used in N-terminal sequence analysis of blocked proteins, one for Nα-pyrrolidone carboxyl-proteins in solution (Basic Protocol 1) or blotted onto a membrane (Alternate Protocol 1) and the other for Nα-acyl-proteins blocked with other acyl groups (Basic Protocol 2, Alternate Protocol 2). The enzyme involved in the first of these reactions, pyroglutamate aminopeptidase (EC 3.4.19.3), works on intact proteins as well as on peptides, and represents an excellent reagent in the unblocking/sequencing arsenal. The Support Protocol describes a colorimetric assay for pyroglutamate aminopeptidase activity. The enzyme used for unblocking N-terminal acetyl/formyl amino acids, acylaminoacyl-peptide hydrolase (EC 3.4.19.1; also referred to as acyl-peptide hydrolase, N-acetyllalanine aminopeptidase, and acylamino acid–releasing enzyme in the literature) is more restricted in that it cannot use intact proteins as substrate, but can be used to obtain N-terminal sequence from peptides containing up to 20 residues. Consequently, any sequencing protocol with this latter enzyme must include fragmentation of the protein before unblocking can be carried out. To avoid extensive purification after fragmentation, newly generated peptides are chemically blocked with either succinic anhydride (Basic Protocol 2) or phenylisothiocyanate/performic acid (Alternate Protocol 2), and hydrolase is applied to the total mixture of peptides, only one of which, the acylated N-terminal peptide, should be a substrate for hydrolase. After incubation, the mixture of peptides is subjected to sequence analysis.

A great variety of chemical methods have been applied to the problem of unblocking N-termally blocked proteins. The methods include acid-catalyzed hydrolysis (Basic Protocol 3) or methanolysis, hydrazinolysis, and β-elimination after acid-catalyzed N-O shift. For each of these methods, a variety of conditions have been adapted to satisfy the unique properties of different blocking groups and different proteins/peptides. For chemical removal of acetyl and longer-chain alkanoyl groups (Alternate Protocol 3), conditions may have to be harsh enough to cause extensive cleavage of, for example, aspartyl peptide bonds. For chemical removal of formyl groups and opening of the cyclic imide of pyrrolidone carboxylate (Alternate Protocol 4), reaction conditions can generally be sufficiently mild that few or no peptide bonds are cleaved. Blocked proteins that are devoid of aspartate and other labile peptide bonds are not often encountered, so the best substrates for chemical unblocking methods are aspartate-free N-terminal peptides. Because the yield of unblocked peptide varies extensively, the amounts of protein recommended for these protocols are considerably larger than those needed for the direct sequencing of an unblocked protein.

It is important to keep in mind that the side chain amides of asparagine and glutamine are among the sensitive amide bonds that are likely to be modified by the various treatments. In general, it should be safe to conclude that even low yields of amide together with high yields of the acid originated as the amide in the acid-treated sequence under study. However, if hydrolysis is extensive enough that the amide cannot be observed at all, the wrong amino acid will obviously be identified.

The choice of method for removing N-terminal blocking groups depends on the nature of the blocking group and the N-terminal amino acid. In some cases information about the groups will be available from the prior history of the sample, from mass analysis, or from studies of homologous proteins. In the absence of such information, the process is trial and error. Testing for hydrazine lability, acid lability, and enzyme susceptibility provides clues as to the nature of the blocking group. Successful removal of the group should make it possible to obtain sequence information beyond the blocked residue.
REMOVAL OF PYRROLIDONE CARBOXYLIC ACID WITH PYROGLUTAMATE AMINOPEPTIDASE TREATMENT OF PROTEINS IN SOLUTION

Pyrrolidone carboxylic acid (PCA), also called pyroglutamic acid (see Fig. 11.7.1), is formed by cyclization of glutamine or glutamic acid to form an amide bond between the α-amino group and the δ-carboxyl group. PCA is found on many naturally occurring proteins and peptides. In addition, PCA may be formed by cyclization of amino-terminal glutamine residues during protein isolation, proteolysis and separation of peptides, and/or protein sequencing (Crimmins et al., 1988). The presence of PCA precludes direct amino-terminal sequence analysis because the α-amino group is not available for reaction with the Edman reagent. The enzyme pyroglutamate aminopeptidase (EC 3.4.19.3) can be used to remove pyroglutamate and leave a free α-amino group on the adjacent residue accessible for Edman degradation and other chemical reactions. The enzyme may be used to digest proteins and peptides either in solution or after electroblotting to membranes (see Alternate Protocol 1).

The following protocol describes enzymatic removal of pyrrolidone carboxylic acid from proteins or peptides in solution. The reaction is generally more efficient in solution than on electroblotted proteins (see Alternate Protocol 1).

Materials

- Protein or peptide, reduced and alkylated (UNIT 11.1)
- PGA digestion buffer (see recipe)
- Calf liver pyroglutamate aminopeptidase (EC 3.4.19.3; sequencing grade, Boehringer Mannheim)
- 0.05 M acetic acid
- Nitrogen (research grade)
- Screw-cap vial
- Magnetic stirring plate

Additional reagents and equipment for dialysis (UNIT 4.4 & APPENDIX 3B)

1. Prepare an 0.5 to 1 mg/ml solution of protein or peptide in PGA digestion buffer.

   Before PGA digestion, the protein should be reduced and alkylated (UNIT 11.1). If the protein preparation contains components other than those specified for PGA digestion buffer, the protein solution should be dialyzed against PGA digestion buffer at 4°C (UNIT 4.4 & APPENDIX 3B) or otherwise desalted.

2. Transfer the protein solution to a screw-cap vial containing a stir bar.

   The vial should have a volume no greater than three times the volume of the sample.
3. Dissolve an aliquot of calf liver pyroglutamate aminopeptidase in water to give a concentration of 0.25 mg/ml (enzyme stock solution).

*Sequencing-grade enzyme is supplied by the manufacturer (Boehringer Mannheim) in vials containing 25 µg enzyme protein. The lyophilized material supplied by the manufacturer contains ~70% (w/w) sucrose, 17% (w/w) potassium phosphate, 8% (w/w) EDTA, and 5% (w/w) enzyme. A single vial should be reconstituted in 0.1 ml water, then flushed with nitrogen and stored ≤1 week at 4°C.

If bulk enzyme (Boehringer Mannheim) is used, reconstitute at 0.25 mg/ml. Some authors report successful storage of this preparation up to 1 month at −20°C in screw-cap vials that have been flushed with nitrogen.

4. Add sufficient enzyme stock solution to the protein solution in the screw-cap vial to give an enzyme/substrate ratio of ~1/300 (w/w). Flush the vial with nitrogen and stir the solution 7 to 9 hr at 4°C.

*Enzyme/substrate ratio and digestion time are dependent on the particular protein or peptide being digested. The rate of removal of PCA varies with the adjacent residue (see Background Information).

*Small peptides may be digested at higher temperatures (40°C) for shorter times, generally 2 to 3 hr (Dimaline and Reeve, 1983).

5. Add a second aliquot of enzyme stock solution (equal in volume to that added in step 4). Flush the vial with nitrogen and stir the solution an additional 14 to 16 hr at room temperature.

6. Dialyze the reaction mixture against 0.05 M acetic acid (*UNIT 4.4 & APPENDIX 3B*). The protein is now suitable for sequence analysis.

*Alternatively, the protein or peptide may be separated from the reagents by any suitable chromatographic or electrophoretic technique (see Chapters 8 and 10). The reaction mix may also be applied directly to the sequencer support.

### REMOVAL OF PYRROLIDONE CARBOXYLIC ACID WITH PYROGLUTAMATE AMINOPEPTIDASE TREATMENT OF ELECTROBLOTTED PROTEINS

Identification of proteins separated by gel electrophoresis is conveniently achieved by performing amino-terminal sequence analysis after electroblotting the proteins onto polyvinylidene difluoride (PVDF) membranes (*UNITS 10.7 & 11.3*). If no sequence is obtained when the blotted protein is sequenced, the amino terminus of the protein may be blocked by pyrrolidone carboxylic acid (PCA) or another modification of the α-amino group. Digestion with pyroglutamate aminopeptidase (EC 3.4.19.3) is useful for removing PCA prior to sequencing.

**Additional Materials** *(also see Basic Protocol 1)*

- Single protein band on PVDF membrane (*UNIT 10.7*) containing ≥50 pmol protein
- 0.5% (w/v) polyvinylpyrrolidone (mol. wt. 40,000, PVP-40; Sigma) in 0.1 M acetic acid
- PGA digestion buffer (see recipe)
- Screw-cap polypropylene microcentrifuge tube

1. Dissolve an aliquot of calf liver pyroglutamate aminopeptidase in water to give a concentration of 0.25 mg/ml (enzyme stock solution).

*Sequencing-grade enzyme is supplied by the manufacturer (Boehringer Mannheim) in vials containing 25 µg of enzyme protein. The lyophilized material supplied by the manufacturer contains ~70% (w/w) sucrose, 17% (w/w) potassium phosphate, 8% (w/w) EDTA, and 5%*
A single vial should be reconstituted in 0.1 ml water, then flushed with nitrogen and stored ≤ 1 week at 4°C.

If bulk enzyme (Boehringer Mannheim) is used, reconstitute at 0.25 mg/ml. Some authors report successful storage of this preparation up to 1 month at −20°C in screw-cap vials that have been flushed with nitrogen.

2. Place single protein band on PVDF membrane in a screw-cap polypropylene micro-centrifuge tube and rinse well with water. Add 0.5% PVP-40 in 0.1 M acetic acid to cover. Incubate 30 min at 37°C.

After rinsing, as much as possible should be drained off without allowing the membrane to dry.

3. Rinse ten times with water.

4. Cover the PVDF membrane with PGA digestion buffer.

5. Estimate the amount (micrograms) of blotted protein present.

The amount of protein present in the band may be estimated from the amount applied to the gel, or, preferably, by comparing the intensity of the stained band with a dilution series of a known protein. An estimate +/−100% is generally adequate.

6. Add enzyme stock solution to achieve an enzyme/substrate ratio of 1/20 (w/w). Flush the vial with nitrogen and close. Incubate 5 hr at 4°C, then 18 hr at room temperature.

7. Rinse the PVDF membrane with water several times. Air dry. The pieces are now suitable for sequence analysis.

COLORIMETRIC ASSAY FOR PYROGLUTAMATE AMINOPEPTIDASE ACTIVITY

Pyroglutamate aminopeptidase activity is not particularly stable. One cause of failure to remove an amino-terminal blocking group may be lack of activity in the enzyme stock solution. The following protocol describes the method used by Boehringer Mannheim and several authors for monitoring enzyme activity based on generation of β-naphthylamine from L-pyroglutamyl-β-naphthylamide. An alternative method for monitoring enzyme activity—assaying for the hydrolysis of a synthetic peptide with ninhydrin—is described in Doolittle (1972).

Materials

PGA digestion buffer (see recipe)
PGA-N substrate solution (see recipe)
25% (v/v) trichloroacetic acid
0.1% (w/v) sodium nitrite
0.5% (w/v) ammonium sulfamate
NED solution (see recipe)
13 × 100–mm test tubes
37°C incubator or water bath

1. Prepare a 13 × 100–mm test tube for each enzyme sample and a “no enzyme” blank. Add 1.0 ml PGA digestion buffer and 0.1 ml PGA-N substrate. Incubate 5 min at 37°C.

The no enzyme blank is prepared identically to the enzyme sample, except that the enzyme solution is omitted from the incubation; it is added after the reaction has been stopped.
2. Add 0.1 ml enzyme stock solution to enzyme sample tube; add nothing to blank tube. Mix well. Incubate exactly 15 min at 37°C.

3. Add 1.0 ml of 25% trichloroacetic acid to all tubes. Add 0.1 ml enzyme stock solution to blank tube. Mix well.

   If precipitate forms, filter through medium-grade filter paper into clean tubes.

4. Transfer 1.0 ml from each tube into a clean tube.

5. Add 1.0 ml of 0.1% sodium nitrite and mix well. Incubate 3 min at 37°C.

6. Add 1.0 ml of 0.5% ammonium sulfamate and mix well. Let stand ~5 min at room temperature.

7. Add 2.0 ml NED solution and mix well. Incubate 20 min at 37°C.

8. Mix samples and read absorption at 578 nm.

9. Use $A_{578}$ to calculate activity (U/ml enzyme solution) using the following formulas:

   $$
   \Delta A_{578} = A_{578, \text{sample}} - A_{578, \text{blank}}
   $$

   Activity = 0.163 \times \Delta A_{578}

**REMOVAL OF ACYLAMINO ACIDS BY HYDROLASE TREATMENT USING SUCCINIC ANHYDRIDE AS BLOCKING REAGENT**

Instead of purifying the N-terminal peptide after fragmentation of a blocked protein, the generated peptides can be rendered inert by chemical blocking using one of two blocking reactions: succinylation with succinic anhydride (as in this protocol) and carbamoylation by reaction with isothiocyanate followed by oxidation with performic acid to convert thiocarbamate to carbamate (Alternate Protocol 2). The general strategy for establishing N-terminal sequences in Nα-acylated proteins is outlined in Figure 11.7.2. All steps—fragmentation of the blocked protein, blocking the newly generated N-termini, treatment with aminoacyl-peptide hydrolase and finally with acylase—are performed in a single test tube to ensure optimum yield. Because the enzyme releases an N-terminal acylamino acid, special considerations must be given to identification of this first residue. Direct identification by HPLC is possible; in the protocol below the acetylamino acid is hydrolyzed with acylase, and the liberated amino acid is identified by direct amino acid analysis. Other protocols involve derivatization with phenacyl bromide and analysis of the resulting phenacylate acetylamino acids by reversed-phase HPLC (Hirano et al., 1993).

Intact protein is digested with specific endoproteases (UNIT 11.1) or cleaved with cyanogen bromide (UNIT 11.4), the newly generated amino groups are blocked, and the N-terminal Ac-peptide is unblocked with acylaminoacyl-peptide hydrolase for sequencing. The blocked N-terminal amino acid is treated with acylase and identified. In this protocol succinic anhydride is used to block newly generated peptides; in Alternate Protocol 2 phenylisothiocyanate and performic acid are used for blocking to yield phenyl carbamates.

*NOTE:* This protocol was developed using an LKB Alpha Plus (ninhydrin-based) Analyzer (Pharmacia Biotech) for amino acid analysis. This analyzer requires $\geq 0.5$ to 1 nmol amino acid for a significant analysis. With more sensitive amino acid analysis methods, the amount of sample can obviously be reduced.
**Materials**

1 nmol/10 µl protein/peptide dissolved in a small volume of suitable volatile solvent  
20% (v/v) acetic acid  
Succinic anhydride  
12% (v/v) triethylamine (TEA)  
20% (v/v) trifluoroacetic acid (TFA)  
Ethyl ether  
Hydrolase buffer I: 50 µM sodium phosphate buffer (pH 7.2; *APPENDIX 2E*)/  
1 mM EDTA/2 mM MgCl₂  
0.5 M NaOH  
Acylaminoacyl-peptide hydrolase (EC 3.4.19.1; Pierce, Takara Biochemical, Boehringer Mannheim, or Sigma)  
70% (v/v) formic acid  
Acylase buffer: 100 mM sodium phosphate buffer, pH 7.0 (*APPENDIX 2E*)  
Acylase I (3.5.1.14; e.g., Sigma)  
0.2 M sodium citrate with pH adjusted to pH 2.2 using concentrated HCl  
0.9 × 7.5–cm glass test tubes  

Additional reagents and equipment for endoprotease digestion (*UNIT 11.1*) or cyanogen bromide cleavage (*UNIT 11.4*), and reversed-phase HPLC (*UNIT 11.6*)

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**Figure 11.7.2** Strategy for the sequencing of Nα-acetylated proteins, unblocking with acylaminoacyl-peptide hydrolase. Symbols: Ac, acetyl group; R, amino acid side chain; X, Y, and Z, amino acid residues.
**Generate peptides**

1. Transfer 1 to 2 nmol protein in a suitable volatile solvent to a 0.9 × 7.5–cm glass test tube and lyophilize.

2. Generate peptides by digesting the protein with an endoprotease (UNIT 11.1) or by cleaving it with cyanogen bromide (UNIT 11.4).

   *For endoprotease digestion, select an endoprotease with defined cleavage. Resuspend the protein in 50 µl of an appropriate buffer and add enzyme to give an enzyme/substrate ratio of 1/20 (w/w). Incubate briefly.*

3. Heat the reaction mixture 5 min at 100°C or acidify it with 20% acetic acid (when carbonate buffers are used) to stop proteolysis.

4. Lyophilize the reaction products.

**Succinylate α- and ε-NH₂ groups**

5. Dissolve lyophilized sample in 50 µl water or carbonate buffer. Add a 200-fold molar excess of solid succinic anhydride in small portions over a span of 1 hr with vigorous mixing on a vortex stirrer. Maintain pH at 10.0 by adding 12% triethylamine.

6. Let the reaction mixture (~pH 10) stand overnight at room temperature.

7. Acidify the reaction mixture to pH 2 to 2.5 with 20% TFA. Lyophilize thoroughly.

8. Extract the residue six to ten times with 2 ml ethyl ether to remove all succinic acid and salts of triethylamine.

   *The combined ether extracts can be air dried and screened for any possible presence of peptides by reversed-phase HPLC on a C18-100/300Å column (UNIT 11.6) and/or by amino acid analysis after hydrolysis.*

**Unblock N-terminal peptide and sequence**


   *The residue is now invisible.*

10. Add 50 µl hydrolase buffer I and adjust the pH to 7.2 with 1 to 3 µl of 0.5 M NaOH.

11. Add 2 to 5 µg acylaminoacyl-peptide hydrolase to the total peptide mixture and incubate 6 hr at 37°C.

   *More enzyme can be used because the enzyme itself is an acetylated protein and hence does not give any background in sequencing. When pure enzyme is used, incubation times ≤12 hr do not give any side reactions. Although the hydrolase is quite active on short peptides, larger quantities of enzyme and longer incubation times may well be needed for longer peptides.*

   *Acetylated peptides with three to eight amino acids are essentially completely hydrolyzed under standard conditions unless they contain positively charged amino acids or proline; longer peptides such as the acetylated 16-residue fibrinopeptide A and the natural 13-residue Ac-α-MSH were hydrolyzed ~50% in 12 hr, but the 14-residue Ac-renin substrate was only hydrolyzed ~2% in 20 hr with 4 µg of hydrolase (Kobayashi and Smith, 1987).*

12. Heat-inactivate the reaction 5 min at 100°C.

13. Lyophilize the sample.

14. Dissolve sample in 50 µl of 70% formic acid. Apply an aliquot equivalent to 0.5 nmol protein to the sequencer to identify the N-terminal sequence from the second residue onwards.
Lysine is detected as the phenylthiohydantoin (PTH) derivative of Nε-succinyl-lysine, which elutes ∼1 min in front of PTH-alanine (about at the same time as Nε-acetyl-lysine) in standard analysis.

Identify the blocked N-terminal amino acid

15. After removing the required aliquot for sequencing, lyophilize the remaining 0.5 to 1.5 nmol protein.

16. Dissolve lyophilized sample in 50 µl acylase buffer. Add 5 U acylase I at pH 7.0 and digest 6 hr at 37°C.

17. Lyophilize the reaction mixture.

18. Dissolve the residue in 50 µl citrate buffer, pH 2.2. Apply entire sample to an amino acid analyzer.

ALTERNATE PROTOCOL 2

REMOVAL OF ACYlamino ACIDS BY HYDROLASE TREATMENT USING PHENYLISOTHIOCYANATE/PERFORMIC ACID AS BLOCKING REAGENTS

This protocol is the basis for a commercially available kit for unblocking proteins (Tsunasawa et al., 1990; Hirano et al., 1993). The catalog/Technical Guide 1 from Takara Biochemical describes kits and methods for chemical and enzymatic removal of blocking groups and sequencing of N-terminally blocked protein. As indicated in Figure 11.7.2, the general strategy in this protocol is essentially the same as that in Basic Protocol 2. The two protocols differ in the chemistry for blocking newly generated peptides.

Additional Materials (also see Basic Protocol 2)

12.5% (v/v) trimethylamine
Phenylisothiocyanate
Benzene
Ethyl acetate
Performic acid, freshly prepared by mixing 95 µl of 99% formic acid and 5 µl of 30% hydrogen peroxide
Hydrolase buffer II: 10 mM sodium phosphate buffer (pH 7.2; APPENDIX 2E)
0.1 mM dithiothreitol (DTT)
0.5% (v/v) trifluoroacetic acid (TFA)
0.9 × 7.5–cm glass test tubes
IEC CL clinical centrifuge or equivalent

Generate peptides

1. Prepare sample and generate peptides by enzymatic or chemical cleavage, and lyophilize the reactions products as described in Basic Protocol 2, steps 1 to 4.

Block α- and ε-NH₂ groups

2. Dissolve lyophilized peptides in 100 µl of 12.5% trimethylamine. Add 10 µl phenylisothiocyanate and mix well. Incubate 1 hr at 50°C.

3. Add 250 µl benzene and mix by vortexing. Centrifuge 5 min at maximum speed in an IEC CL centrifuge (5000 rpm). Remove the upper layer. Extract the lower phase two more times with benzene.

4. Similarly, extract the remaining lower phase three times with 250 µl ethyl acetate.
5. Lyophilize the final lower phase.

6. Repeat steps 2 to 5 once.

7. Dissolve lyophilized sample in 50 µl performic acid and mix well. Incubate 1 hr at 0°C, to convert the thiocarbamate to stable phenyl carbamate.

8. Remove the reagent under reduced pressure in a vacuum desiccator. Dissolve the residue in 100 µl cold water and lyophilize repeatedly to ensure complete removal of organic solvents.

**Unblock N-terminal peptide**

9. Dissolve lyophilized blocked peptides in 50 µl hydrolase buffer II. Add 2 to 5 µg acylaminoacyl-peptide hydrolase to the total peptide mixture. Incubate 6 hr at 37°C.

   *The lyophilized peptides are not visible in the tube. If there is any difficulty in dissolving the substrate, sonicate the sample.*

   *The authors recommend 0.05 to 1.0 U of commercial acylaminoacyl-peptide hydrolase per nmol of substrate in their kit.*

10. Heat inactivate the mixture 5 min at 100°C.

11. Lyophilize the reaction mixture.

**Sequence the peptide**

12. Dissolve reaction products in 50 µl of 0.5% TFA (or other suitable solvent). An appropriate aliquot may now be applied to the glass-fiber disc of a sequencer.

### REMOVAL OF ACETYL GROUPS BY ACID HYDROLYSIS

The protein is digested with endoprotease (see Table 11.1.3) to release peptides with a minimum of acid-labile peptide bonds. The preferred method for preparing peptides is to digest the protein with the protease from *Staphylococcus aureus* strain V8 (endoproteinase Glu-C), which cleaves peptide bonds at the carboxyl side of aspartate and glutamate in phosphate buffer, pH 7.8 (*UNIT 11.1*). With this cleavage, the aspartyl bond (generally the most labile peptide bond) is at the C-terminus, and, at worst, acid treatment will give a high background of aspartate in the first cycle only, with relatively little interference in subsequent cycles. Peptide purification using either ion-exchange chromatography (*UNIT 8.2*) or high-performance liquid chromatography (HPLC) can be quite tedious because of the complex assay for the blocked N-terminal peptide—the blocked peptide is identified after complete enzymatic digestion with pronase and treatment of the resulting acylamino acid with acylase to release free amino acid (Chin and Wold, 1985, 1986). Because of the anticipated low yield of pure N-terminal peptide, the amount of starting protein required is about five times greater than the amount of peptide analyzed. Blocked peptides are hydrolyzed with HCl, lyophilized, and sequenced.

### Materials

- 2 to 5 nmol blocked peptide
- 1 N HCl
- 70% (v/v) formic acid or other suitable solvent
- 1-ml glass vial
- 14-oz. propane torch
- 110°C oven

1. Transfer 2 to 5 nmol blocked peptide to a 1-ml vial and lyophilize.
2. Add 200 to 500 µl of 1 N HCl and seal the vial using a 14-oz. propane torch. Incubate 10 min in 110°C oven.

*The incubation time may have to be adjusted for each individual peptide. The yield of unblocked N-terminus increases with time, but background from peptide bond cleavage also increases to complicate sequence determination. Based on the results with a limited number of proteins, 10 to 20 min appears to be optimal.*

3. Immediately cool the vial on ice. Break the seal and lyophilize the hydrolysate.

4. Dissolve sample in 40 to 100 µl of 70% formic acid. An appropriate aliquot (e.g., 10 to 20 µl, or 0.5 to 1.0 nmol peptide) can now be applied to the sequencer.

*As a solvent, 70% formic acid has been found to be uniformly useful, especially for small amounts of material of low solubility in water. Additional exposure to acid does not appear to affect results. Also, 0.5% (v/v) trifluoroacetic acid (TFA) is a good solvent.*

*Because unblocking is likely to be incomplete, the amount of material subjected to sequencing is increased.*

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**ALTERNATE PROTOCOL 3**

**REMOVAL OF ACETYL GROUPS FROM N-TERMINAL SER AND THR WITH ANHYDROUS TRIFLUOROACETIC ACID**

Treatment of proteins containing N-terminal Ac-Ser or Ac-Thr with anhydrous trifluoroacetic acid causes an N→O acyl shift and β-elimination of the acetyl esters. This procedure is adapted from Wellner et al., 1990.

**Materials**

- Polybrene solution: 100 mg/ml Polybrene in 6.7 mg/ml NaCl (0.115 M final)
- 0.1 nmol/µl protein or peptide in a suitable volatile solvent
- Anhydrous trifluoroacetic acid (TFA)
- 12-mm-diameter glass-fiber filter disc, treated with TFA (standard sequencer disc, Applied Biosystems)
- 1.5-ml polypropylene microcentrifuge tube with cap
- 45°C and 65°C oven

1. Place a 12-mm glass-fiber filter disc in a 1.5-ml polypropylene microcentrifuge tube. Wet disc with 30 µl polybrene solution and dry.

2. Apply 10 to 20 µl protein or peptide solution to the filter disc and dry again.

3. Add 30 µl anhydrous TFA to the thoroughly dried filter. Close tube and incubate 4 min at 45°C.

4. Open the tube in a fume hood and let stand ~5 min to allow most of the TFA to evaporate.

5. Incubate open tube 10 min at 45°C. Close tube and incubate 16 hr at 65°C or 3 days at 45°C. The filter can now be applied to the pad of a sequencer.
REMOVAL OF N-TERMINAL FORMYL GROUPS AND UNBLOCKING OF PYRROLIDONE CARBOXYL GROUPS WITH ANHYDROUS HYDRAZINE

Protein blocked at the N-terminus is incubated in the presence of anhydrous hydrazine to remove formyl groups and open the cyclic imide of pyrrolidone carboxylate; it is then used for sequencing. In treatment with hydrazine, asparagine and glutamine are converted to the corresponding hydrazides and arginine is converted to ornithine, so three additional PTH standards are required to identify these amino acids in the sequence. This procedure is taken from Miyatake et al., 1993.

**Materials**

~100 pmol protein, free or bound to polyvinylidene difluoride (PVDF) membrane
3-phenyl-2-thiohydantoin (PTH) derivatives of β-hydrazidyl-aspartate, γ-hydrazidyl-glutamate, and ornithine (standards)
Anhydrous hydrazine
4 × 44–mm (small) test tubes
13 × 100–mm (large) test tubes

1. Place ~100 pmol protein in a 4 × 44–mm test tube and dry.

   *The sample may be protein on a membrane removed from a sequencer after obtaining evidence that the sample is blocked.*

2. Place small test tube in a 13 × 100–mm test tube that contains 100 µl anhydrous hydrazine. Seal the large test tube under a vacuum.

3. Incubate the sample 8 hr at −5°C or 4 hr at 20°C.

   *Treatment at −5°C is sufficient to remove formyl blocking groups, and treatment at 20°C is sufficient to open the cyclic imide of pyrrolidone carboxylate. Under the latter conditions, removal of acetyl blocking groups appears to be very low (<10%). At the lowest temperature, side reactions are minimal; at 20°C, only the most labile peptide bonds may be cleaved, but the side-chain amides of asparagine and glutamine are converted to the corresponding hydrazides, and arginine is converted to ornithine. The two conditions can be used in succession if the first one fails. It should be noted that the PTH derivatives of the two hydrazides elute well after leucine in a standard HPLC program; they may not be observed unless the elution time is extended.*

4. Open the tube and dry 16 hr in a vacuum desiccator. The treated sample may now be sequenced.

**REAGENTS AND SOLUTIONS**

*Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see APPENDIX 2E; for suppliers, see SUPPLIERS APPENDIX.*

**NED solution**

Dissolve 25 mg N-(naphthyl)-ethylenediamine-2 HCl (Sigma; 2 mM final) in 50 ml absolute ethanol. Prepare fresh daily.

**PGA digestion buffer**

0.1 M NaHPO₄, pH 8.0
5 mM dithiothreitol (DTT)
10 mM EDTA
5% (v/v) glycerol
Cool to 4°C

*Prepare fresh before use.*
**PGA-N substrate**

Dissolve 5.5 mg L-pyroglutamyl-β-naphthylamide (Sigma; 0.02 M final) in 1 ml methanol. Prepare fresh daily.

**COMMENTARY**

**Background Information**

Of the approximately 200 covalent modifications known to exist in proteins, the sixteen shown in Table 11.7.1 involve the N-terminus (Krishna and Wold, 1993), and all but one of these result in “blocked” N-termini that cannot be sequenced by regular Edman sequencing. The exception is acylation of the N-terminus with another amino acid, leaving a free N-terminus in which the original N-terminal amino acid has become the second residue in the sequence. Blocked N-termini have represented a major hurdle to investigators who wish to establish the N-terminal sequence of a protein. A good deal of effort has been expended in establishing methods that can be used to undo the blocking reaction and make the protein or peptide susceptible to Edman sequencing. Methods for unblocking include both chemical and enzymatic reactions. The choice of method depends on the nature of both the blocking group and the blocked N-terminal amino acid. In these protocols only the two most common blocking structures, pyrrolidone carboxyl and acetylaminoacyl terminals, are considered in detail; others (listed in Table 11.7.1) are mentioned briefly.

Enzymatic methods for unblocking N-termini are more specific than chemical methods. When it was first established that all prokaryotic proteins started with an initial N°-formyl-Met, the search for the enzyme(s) responsible for the removal of the formyl (or the formyl-Met) group was immediately started, and a formyl-hydrolase was found. The occurrence of proteins with an N-terminal formyl group still attached is rare, and the reaction catalyzed by this enzyme has not become a part of the standard sequencing procedures. When it was subsequently found that a large number of eukaryotic proteins contained acylated N-termini, a similar search for acyl-hydrolases was initiated. In spite of much work in this area, no such enzyme has yet been found. Considering that initiation of protein synthesis in eukaryotes involves free Met and that any acylated derivative is thus made co- or post-translationally, presumably for a specific purpose, the absence of the sought-after unblocking hydrolases is perhaps not surprising. Similarly, there is no enzyme known to catalyze the hydrolysis (deacylation) of the cyclic amide of pyrrolidone carboxylate to convert this N-terminal blocking group to glutamate. However, there are

<table>
<thead>
<tr>
<th>Derivative</th>
<th>No. carbon atoms</th>
<th>Reference</th>
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<tr>
<td>N°-formyl</td>
<td>C1</td>
<td>Capecchi (1966)</td>
</tr>
<tr>
<td>N°-acyethyl</td>
<td>C2</td>
<td>Arfin and Bradshaw (1988)</td>
</tr>
<tr>
<td>N°-acyl</td>
<td>C2, C3, C6, C8, C10</td>
<td>Neubert et al. (1992)</td>
</tr>
<tr>
<td>N°-myristoyl</td>
<td>C14</td>
<td>Carr et al. (1982)</td>
</tr>
<tr>
<td>N°-lauroyl</td>
<td>C12</td>
<td>Moscarello et al. (1992)</td>
</tr>
<tr>
<td>N°-tetradeca(mono and di)enoyl</td>
<td>C14:1, C14:2</td>
<td>Moscarello et al. (1992)</td>
</tr>
<tr>
<td>N°-pyrrolidone carboxyl</td>
<td>—</td>
<td>Orlowski and Meister (1971)</td>
</tr>
<tr>
<td>N°-aminoacyl</td>
<td>—</td>
<td>Kaji (1976)</td>
</tr>
<tr>
<td>N°-αketoacyl</td>
<td>—</td>
<td>Recsei and Snell (1984)</td>
</tr>
<tr>
<td>N°-glucuronyl</td>
<td>—</td>
<td>Kolattukudy (1984)</td>
</tr>
<tr>
<td>N°-methyl</td>
<td>—</td>
<td>Siegel (1988)</td>
</tr>
</tbody>
</table>

*Taken from Krishna and Wold (1993).
enzymes that will remove the entire blocked amino acid. One of these, pyroglutamate aminopeptidase (5-oxopropyl peptidase; Orłowski and Meister, 1971), catalyzes removal of pyrrolidine carboxylate (pyroglutamate) from the N-terminus of proteins and peptides, and, as illustrated in the protocols in this unit, is being used very effectively to unblock proteins for sequencing. Another type of enzyme, acylaminoacyl-peptide hydrolase, catalyzes the removal of other acylamino acids from the N-terminus. In both cases, the acylated N-termini are unblocked by removing the entire N-terminal acylamino acid, leaving residue 2 as the new free N-terminus.

Pyroglutamate is found at the amino terminus of a number of naturally occurring proteins. In some cases the cyclized residue has been found to be essential for biological activity, but in other cases, such as in immunoglobulin chains, formation of pyrocarboxylic acid (PCA) does not appear to affect function (Abraham and Podell, 1981). Pyroglutamate can also form by cyclization of glutamine or glutamic acid during protein purification and sequencing. This cyclization is more likely to occur under acid conditions (Crimmins et al., 1988). The presence of PCA precludes amino-terminal sequence analysis by the widely used Edman degradation method.

The enzyme pyroglutamate aminopeptidase is present in a wide variety of plant and animal tissues and in numerous species of bacteria (Szewczuk and Kwiatkowska, 1970; McDonald and Barrett, 1986). Although the first preparations used for deblocking peptides prior to sequencing were isolated from bacteria (Doolittle and Armentrout, 1968), the present commercially available enzymes are of mammalian origin.

The enzyme acylaminoacyl-peptide hydrolase (see Basic Protocol 2 and Alternate Protocol 2) is most active with short peptides containing acetylated alanine, methionine, glycine, serine, or threonine as the N-terminus. It will also remove formyl-, propionyl-, and butyryl derivatives of these amino acids, albeit at a slower rate than for the acetyl derivatives (Gade and Brown, 1987). It is not known whether longer-chain alkanoyl derivatives are also acceptable as substrates; as more proteins are found with N-terminal saturated and unsaturated fatty acyl groups, the suitability of these derivatives as substrates for the hydrolase becomes increasingly relevant. In addition to peptide length, the nature of the amino acids in the vicinity of the N-terminus also affects enzyme activity (Krishna and Wold, 1992; Sokolik et al., 1994). An immobilized derivative of the enzyme has been prepared, making it more stable and reusable (Farries et al., 1991). As in the case of the chemical methods, definition of a specific set of unblocking and sequencing conditions may have to be modified empirically to give optimal information yield for a particular protein.

Many of the methods for unblocking proteins use simple chemical reagents; these methods have evolved to take advantage of the unique chemical properties of each derivative. Thus, specific unblocking of acetyl-serine or acetyl-threonine can be effected through a putative β-elimination after N-O-acetyl shift in anhydrous acid (Wellner et al., 1990), pyruvoyl can be converted to alanine by reductive amination (Huynh et al., 1984), and formyl and pyrrolidone carboxylate groups can be removed by mild treatment with acid or hydrazine under conditions where acetyl and longer-chain acyl groups are not cleaved (Miyatake et al., 1993). It is possible to achieve preferential cleavage of acetyl blocking groups as well (see Basic Protocol 3 and Alternate Protocol 3). The main difficulty with chemical unblocking methods is that, in the typical protein, there will always be some particularly labile peptide bonds that may be cleaved by the treatment; in fact, the various acid treatments that have been used to remove N-terminal acetate are typically equal to or harsher than those used to specifically cleave aspartate peptide bonds (Schultz, 1967; Landon, 1977). Because of this, most of the chemical methods are more reliable and specific if they can be applied to short blocked peptides, selected to be void of aspartate if possible, rather than to the intact parent proteins. This in turn means that potentially complex protein fragmentation and peptide isolation steps (Units 11.1-11.6) must be added to the general unblocking procedure.

A number of strategies have been explored to produce pure blocked N-terminal peptides. The most obvious one is to digest the protein with trypsin followed by carboxypeptidase B to remove all C-terminal lysines and arginines in the resulting peptides. In this reaction mixture, all the peptides except the N-terminal one will have the positive charge of the N-terminus; hence, these charged peptides can be retarded on a cation-exchange column, and the N-terminal blocked peptide can be found in the unretarded fraction (Chin and Wold, 1986). Because of all the variable and unique properties of individual proteins, it is necessary to
empirically develop an approach suitable to each protein. The protocols given in this unit are based on a limited number of cases, but they should provide useful starting points for development of appropriate reactions for a particular protein.

**Critical Parameters**

*Removal of pyrrolidone carboxylic acid*

Pyroglutamate aminopeptidase activities isolated from all sources are thiol exopeptidases. The enzyme requires the presence of thiols for activation, so dithiothreitol (DTT) is included in the digestion buffer. The enzyme is inhibited by thiol-binding reagents such as iodoacetate, chloromercuribenzoate, Hg$^{2+}$, Cu$^{2+}$, Co$^{2+}$, and Zn$^{2+}$ (Szewczuk and Kwiatkowska, 1970). The pH optimum of the enzyme is $\sim 8.0$.

Pyroglutamate aminopeptidase is sensitive to denaturing agents. Urea may be used at concentrations $\leq 1$ M, but 0.5 M guanidine hydrochloride decreases enzyme activity to 79% of control values, and the enzyme is essentially inactivated at higher concentrations (Boehringer-Mannheim product information).

**Enzyme specificity**

Pyroglutamate aminopeptidase is specific for pyrrolidone carboxylic acid (PCA) residues at the amino terminus of peptides and proteins. The rate of removal of PCA is dependent on the adjacent residue and the enzyme does not cleave PCA-proline (McDonald and Barrett, 1986).

Highly purified pyroglutamate aminopeptidase should remove only the amino-terminal PCA residue; however, cleavage after an internal proline has been reported (Nagasawa et al., 1988). Early preparations often contained contaminating endoproteases which produced unwanted internal cleavages. When using such preparations, it was essential to repurify the desired intact protein after digestion. The protein-sequencing grade of pyroglutamate aminopeptidase presently available from Boehringer Mannheim is subjected to a quality control test designed to detect contaminating proteases. Although this should eliminate the problem, the prudent investigator will continue to be alert for evidence of additional cleavages.

*Removal of N\$^\alpha\$-acetylamino acids*

It is well established that persistent buffering capacity in the sample to be sequenced seriously jeopardizes the effectiveness of sequencing cycles. For this reason, one of the main concerns in the various unblocking procedures is ensuring that they do not leave an excessive amount of buffers to interfere with sequencing. The two major trouble-makers in these protocols are the succinate used in blocking peptides (Basic Protocol 2), and the phosphate used in enzymatic unblocking (Basic Protocol 2 and Alternate Protocol 2). Once aware of these potential problems, it is relatively simple to develop methods to avoid them. Either extraction and repeated lyophilization are recommended after succinylation and appear to be sufficient to eliminate interfering succinate from the sample. Using small volumes and low concentrations of phosphate buffer in enzymatic unblocking renders interference from phosphate insignificant. It is important to be aware of these potential hazards from introduced impurities to ensure that a successfully unblocked protein or peptide yields meaningful sequence information.

**Troubleshooting**

To the extent that unknown proteins really are unknowns, most of the protocols are strictly empirical and essentially constitute a series of troubleshooting steps. The proposed sequential exploration of hydrazine-labile, acid-labile, and enzyme-susceptible N-termini (Hirano et al., 1993), for example, is designed specifically as a progressive hunting expedition to identify the N-terminus and get sequence information beyond it. Similarly, for unknown blocked proteins, selection of appropriate unblocking procedures, choice of proper enzyme to cause fragmentation, and identification of conditions for the unblocking reaction must be made on a trial-and-error basis. When the amount of protein is limited, this is obviously not an attractive approach. For proteins whose mRNA sequence has been determined, and for which the experiments are designed to explore the extent of N-terminal processing of a given predicted structure, selection of the proper methods can be much more direct.

Pyroglutamate digestion is generally used after a sequencing attempt on a protein or peptide fails to yield sequence information, suggesting that the amino terminus is blocked. The protein is then digested with pyroglutamate aminopeptidase and a second sequencing attempt is made. A second failure to obtain amino-terminal sequence data may indicate either that the blocking group is not pyroglutamate or that enzymatic digestion has not been successful. Strategies for removing other...
blocking groups are discussed below. Several controls to demonstrate enzyme activity of the pyroglutamate aminopeptidase may be employed. Pyroglutamate aminopeptidase activity is fairly labile and sensitive to various inhibitors. Activity of the enzyme may be tested by the colorimetric method described in the Support Protocol. An internal control using a commercially available PCA-containing peptide such as bombesin may be added to the reaction. After digestion, the mixture is separated chromatographically and the peptide subjected to sequence analysis. Crimmins et al. (1988) and Dimaline and Reeve (1983) describe digestion of bombesin and other peptides and separation of unblocked from blocked forms.

If the enzyme is active in the protein mixture, variations in enzyme/substrate ratio and digestion times should be investigated. The rate of cleavage of PCA is dependent on the nature of the adjacent residue. If the rate is low, increasing digestion time or temperature may be necessary. Additional aliquots of enzyme may be added at intervals during digestion to compensate for enzyme lability.

If the substrate protein is large with substantial tertiary structure, the amino terminus may be inaccessible to the enzyme. The protein should be reduced and alkylated before digestion with pyroglutamate aminopeptidase. Denaturing the protein by making a concentrated solution in 8 M urea or 6 M guanidine HCl and then diluting to ≤1 M urea or ≤0.5 M guanidine HCl may be helpful in exposing the amino terminus. It may be necessary to cleave the protein with an endoprotease and isolate the amino-terminal peptide before digestion with pyroglutamate aminopeptidase. Digestion of small peptides tends to occur more easily than digestion of large proteins.

If more than one residue is exposed at the amino terminus, it may indicate that the enzyme preparation contains additional endoprotease activities. A purer preparation of enzyme may alleviate this problem. Alternatively, the digestion products may be separated chromatographically before sequence analysis. Two amino-terminal residues have also been detected when the residue adjacent to the original PCA is a glutamine (Crimmins et al., 1988). Presumably some of this second glutamine cyclized to PCA, which was then removed by the enzyme.

**Anticipated Results**

With Basic Protocol 1, 200 pmol protein should yield 40 to 100 pmol unblocked protein ready for sequence analysis. Digestion of electroblotted proteins (Alternate Protocol 1) is somewhat less efficient but fewer sample-handling steps are required. Depending on the sensitivity of the protein sequencer, 50 pmol starting protein should produce a detectable signal of 10 to 20 pmol.

When combining treatment with hydrolase and proteolysis (Basic Protocol 2 and Alternate Protocol 2), the results can be quite variable because the activity of the hydrolase depends on peptide structure. In a test of eight known acetylated proteins, 500 pmol starting material produced sequences as short as two residues and as long as twelve residues. Under optimized conditions for acid hydrolysis (Basic Protocol 3), 40% to 60% of total peptide yields unblocked N-terminal residues. Treatment with trifluoroacetic acid (Alternate Protocol 3) has given yields of 3% to 40% unblocked N-terminals. Treatment with hydrazine (Alternate Protocol 4) has yielded 50% to 80% unblocking of formylated proteins and 69% to 90% of pyrrolidone-carboxylated proteins.

**Time Considerations**

For Basic Protocol 1 with a reduced and alkylated protein sample that can be placed directly into PGA digestion buffer, setting up the digestion takes ~30 min and should be started early in the morning so the first digestion step, which requires 7 to 9 hr, can be carried out during the remainder of the working day; the second digestion is carried out overnight. Dialysis requires 3 to 4 hr; alternatively, reversed-phase desalting combined with isolation of the blocked protein can generally be accomplished in 60 to 90 min by reversed-phase HPLC. In Alternate Protocol 1, which starts with a blotted protein, preparation and preliminary incubation requires ~90 min and digestion requires 23 hr. Support Protocol 1 takes ~3.5 hr to complete.

Treatment with hydrolase combined with proteolysis (Basic Protocol 2 and Alternate Protocol 2) requires 1 to 2 days depending on the method for blocking newly generated amino groups. Acid hydrolysis (Basic Protocol 3) can be completed in 0.5 day. Treatment with trifluoroacetic acid (Alternate Protocol 3) requires 2 to 4 days, and treatment with hydrazine (Alternate Protocol 4) takes 1.5 days.
**Blocking Groups**

**N-Terminal**

**Supplement 3 Current Protocols in Protein Science**


**Key References**

Chin and Wold, 1986. See above.  
*Describes chemical unblocking using aqueous acid.*

Crimmins et al., 1988. See above.  
*Describes digestion of peptides and a cation-exchange method for isolating digestion products.*

Dimaline and Reeve, 1983. See above.  
*Describes digestion of small amounts of peptides and a reversed-phase HPLC method for monitoring digestion and isolating digestion products.*

Hirano et al., 1993. See above.  
*A general overview of unblocking.*


*Describes the use of hydrolase with succinic acid blocking.*

Miyatake et al., 1993. See above.  
*Describes chemical unblocking using hydrazine.*

*Describes enzymatic unblocking of electroblotted proteins.*

Podell, D.N. and Abraham, G.N. 1978. A technique for the removal of pyroglutamic acid from the amino terminus of proteins using calf liver pyro- 
*Describes the basic method for digestion of milligram quantities of protein.*

Tsunasawa et al., 1990. See above.  
*Describes hydrolysis with phenylcarbamate blocking.*

*Describes digestion and subsequent sequencing of microgram quantities of protein.*

Wellner et al., 1990. See above.  
*Describes chemical unblocking with anhydrous acid.*

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