12

Posttranslational Modifications Analyzed by Automated Protein Ladder Sequencing

Rong Wang and Brian T. Chait

1. Introduction

Protein ladder sequencing *(1)* is a method for obtaining information concerning the primary structures of proteins. The method involves two steps. In the first step, a nested set of fragments of the polypeptide chain is generated by controlled stepwise chemical degradation at the amino terminus (Fig. 1). In the second step, the molecular masses of the set of peptides are measured by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) *(2)*. This measurement results in a mass spectrum that provides information on the sequence and identities of amino acid residues, both unmodified and modified.

The identities of amino acid residues are determined by the mass differences between consecutive peaks in the mass spectrum, and the sequence is defined by their order of occurrence. Posttranslational modifications introduce changes in the masses of the amino acid residues that are modified. Measurement of the molecular masses of such modified residues provides information on the site and nature of the modifications. For example, phosphorylation of serine, threonine, or tyrosine will increase the molecular mass of the modified amino acid residue by 80 Da, whereas deamidation of asparagine or glutamine will decrease the molecular mass by 1 Da.

There are several ways to generate sets of peptide fragments suitable for protein ladder sequencing *(1,3–5)*. This chapter describes a method which uses an automatic gas-phase protein sequencer to produce protein sequencing ladders by controlled partial Edman degradation (*see* Note 1). The resulting peptide ladders are used for identifying posttranslational modifications.

From: *Methods in Molecular Biology, Vol. 61: Protein and Peptide Analysis by Mass Spectrometry*
Edited by: J. R. Chapman Humana Press Inc., Totowa, NJ

```
Starting peptide:    SRASS----------
          Step 1      RASS----------
          Step 2       ASS----------
          Step 3        SS----------
          Step 4         S----------
```

Fig. 1. Nested set comprising starting peptide and four fragments generated by stepwise partial degradation.

2. Materials

2.1. Instruments

1. Sequencer: An ABI model 471A protein sequencer (Applied Biosystems, a Division of Perkin-Elmer Corporation, Foster City, CA) is used in our laboratory to perform the ladder-generating degradation reactions. Other models of automated protein sequencer should also be suitable for this purpose.
2. Mass spectrometer: MALDI-TOF mass spectrometer *(6)* (*see* Note 2).

2.2. Chemical Reagents

2.2.1. Peptide-Supporting Membrane

Immobilon-CD transfer membrane (Millipore, Bedford, MA).

2.2.2. Sequencing Reagents

1. 5% Phenyl isothiocyanate (PITC) in *n*-heptane (R1).
2. 12.5% Trimethylamine (R2).
3. Trifluoroacetic acid (TFA) (R3).
4. Ethyl acetate (S2).

Items 1–4 are obtained from Applied Biosystems and used without further purification.

2.2.3. Mass Spectrometric Reagents and Solvents (see Note 3)

1. Matrix: α-cyano-4-hydroxycinnamic acid *(7)* (Aldrich, Milwaukee, WI).
2. Methanol (Fisher Scientific, Pittsburgh, PA).
3. TFA (Applied Biosystems).
4. Acetonitrile (Applied Biosystems).
5. Water.

3. Methods

3.1. Measuring the Molecular Mass of the Peptide to Be Sequenced

The molecular mass of the peptide (*see* Note 4) is measured by MALDI-TOFMS (*see* Note 5).

1. Prepare a saturated matrix solution of α-cyano-4-hydroxycinnamic acid in water/acetonitrile (2:1 [v/v]), containing 0.1% TFA.
2. Mix the peptide solution with the matrix solution to give a final peptide concentration of 1 μ*M* (*see* Note 6).
3. Apply a small volume (0.5 μL) of the resulting solution onto the sample probe, and dry at room temperature.
4. Insert the sample probe into the mass spectrometer and acquire a mass spectrum in positive- (or negative-) ion mode.

3.2. Peptide Immobilization

The degradation reaction is carried out on peptides that have been immobilized on an Immobilion-CD membrane.

1. Cut the membrane into small pieces: 2 × 2 mm^2 for peptide amounts in the range 1–10 pmol and 5 × 5 mm^2 for peptide amounts >10 pmol.
2. Prior to use, precondition the membrane with methanol/12.5% trimethylamine (1:1 [v/v]) (2 μL for the 2 × 2 mm^2 membrane or 5 μL for the 5 × 5 mm^2 membrane).
3. While the membrane is still moist, apply the peptide sample (1–50 pmol in a volume of 2–5 μL) onto the membrane and dry under a nitrogen flow (*see* Note 7).
4. Load the membrane containing the peptide into the reaction cartridge of the protein sequencer in readiness for the degradation reaction.

3.3. Peptide Ladder Generation

The peptide degradation reaction is carried out in an ABI 471A protein sequencer. A modified sequencing cycle (Table 1) is used to produce the peptide sequencing ladder. Partial degradation is achieved by using a short coupling-reaction time (3 min).

The sequencing program ("run file") consists of two cycle subprograms ("cycle files"), viz. the preconditioning cycle (Table 2) and the partial degradation cycle (Table 1). The preconditioning cycle is used only once to remove residual air from the membrane. Following the preconditioning cycle, the partial degradation cycle is repeated the desired number of times to produce the ladder peptides.

In the partial degradation cycle program (Table 1), the coupling reaction is achieved in steps 1–10 at 55°C. The coupling reaction is followed by washing three times with ethyl acetate (steps 12–21) to remove nonreacted PITC and side products. The cleavage reaction is carried out in steps 23–27 at 46°C (*see* Notes 8 and 9). After cleavage, several steps are used to clean TFA from the system. Steps 11 and 36 set the temperature of the reaction cartridge (*see* Note 10).

3.4. Peptide Mixture Extraction

1. After completing the desired number of cycles of the degradation reaction, withdraw the sample-containing membrane from the sequencer, cut it into small pieces (about 1 × 1 mm^2), and transfer these into a 0.6-mL microcentrifuge tube.

Table 1
Partial Degradation Cycle
Used for Generating Peptide Sequencing Ladder

Step	Cartridge function	Flask function	Parameter	Elapsed time
1	4 PREP R2	39 WAIT	5	00:00:05
2	5 DELIV R2	39 WAIT	10	00:00:15
3	1 PREP R1	39 WAIT	5	00:00:20
4	2 DELIV R1	39 WAIT	2	00:00:22
5	29 DRY CART	39 WAIT	5	00:00:27
6	5 DELIV R2	39 WAIT	90	00:01:57
7	2 DELIV R1	39 WAIT	2	00:01:59
8	29 DRY CART	39 WAIT	5	00:02:04
9	5 DELIV R2	39 WAIT	83	00:03:27
10	29 DRY CART	39 WAIT	10	00:03:37
11	31 RXN HEATER	39 WAIT	46	00:04:23
12	13 PREP S2	39 WAIT	5	00:04:28
13	14 DELIV S2	39 WAIT	9	00:04:37
14	39 WAIT	39 WAIT	5	00:04:42
15	29 DRY CART	39 WAIT	5	00:04:47
16	14 DELIV S2	39 WAIT	9	00:04:56
17	39 WAIT	39 WAIT	5	00:05:01
18	29 DRY CART	39 WAIT	5	00:05:06
19	14 DELIV S2	39 WAIT	9	00:05:15
20	39 WAIT	39 WAIT	5	00:05:20
21	29 DRY CART	39 WAIT	120	00:07:20
22	39 WAIT	39 WAIT	95	00:08:55
23	7 PREP R3	39 WAIT	5	00:09:00
24	9 LOAD R3	39 WAIT	8	00:09:08
25	29 DRY CART	39 WAIT	5	00:09:13
26	39 WAIT	39 WAIT	60	00:10:13
27	29 DRY CART	39 WAIT	5	00:10:18
28	15 LOAD S2	39 WAIT	5	00:10:23
29	30 BLK FLUSH	39 WAIT	5	00:10:28
30	14 DELIV S2	39 WAIT	9	00:10:37
31	39 WAIT	39 WAIT	5	00:10:42
32	29 DRY CART	39 WAIT	5	00:10:47
33	14 DELIV S2	39 WAIT	9	00:10:56
34	39 WAIT	39 WAIT	5	00:11:01
35	29 DRY CART	39 WAIT	5	00:11:06
36	31 RXN HEATER	39 WAIT	55	00:12:01
37	29 DRY CART	39 WAIT	140	00:14:21

Table 2
Preconditioning Cycle
Used to Generate Peptide Sequencing Ladder

Step	Cartridge function	Flask function	Parameter	Elapsed time
1	29 DRY CART	39 WAIT	30	00:00:30
1	4 PREP R2	39 WAIT	5	00:00:35
2	5 DELIV R2	39 WAIT	10	00:00:45

2. Extract the peptide mixture (sequencing ladder) with 10 µL of 2.5% TFA in acetonitrile/water (6:4 [v/v]) for 3 min (with sonication; *see* Note 11).
3. Remove the membrane and add the peptide solution to the matrix solution (*see* Section 3.5. and Note 12).

3.5. Measuring the Molecular Masses of the Ladder Peptides

1. Mix 1–2 µL of the extracted peptide solution with 2 µL of the matrix solution (*see* Sections 2.2.3. and 3.1.).
2. Apply 1 µL of this peptide/matrix solution on the sample probe tip, and dry at room temperature.
3. Acquire mass spectra in the positive-ion mode using a MALDI-TOF mass spectrometer. It is important to note that the mass spectrometric response is dependent on the amino acid composition of the ladder peptides (*see* Note 13).

3.6. Read-Out of the Amino Acid Sequence and the Amino Acid Modifications

The amino acid sequence is determined by calculating the mass differences between consecutive peaks. In the example shown in Fig. 2A, peak (a) corresponds to a mass of 977.2 Da (*see* Note 14) and peak (b) corresponds to a mass of 1064.3 Da. The mass difference is calculated as 1064.3 – 977.2 = 87.1 (Da). The identity of this amino acid residue (Ser) is determined by comparing the calculated mass difference with the amino acid residue masses listed in Table 3 (*see* Note 15). In a similar manner, the mass differences between the other pairs of adjacent peaks are 87.1 (Ser), 71.2 (Ala), 156.3 (Arg), 87.0 (Ser), and so on. The amino acid sequence (N-terminal to C-terminal) is determined by the order of the residues identified from the sequencing ladder—in the direction of high to low mass (i.e., ... SRASS ...).

Modified amino acid residues can be identified using the same procedure as that just described (*see* Note 16). In the example shown in Fig. 2B, the mass difference between (b) and (c), 1231.5 – 1064.3 = 167.2, does not correspond to any of the amino acid residue masses given in Table 3, indicating that the amino acid is modified. The identification of the modification can be made by reference to Table 4 (p. 168). In the present example, the modification is unambiguously identified as phosphorylation of a serine residue (pS). The mass dif-

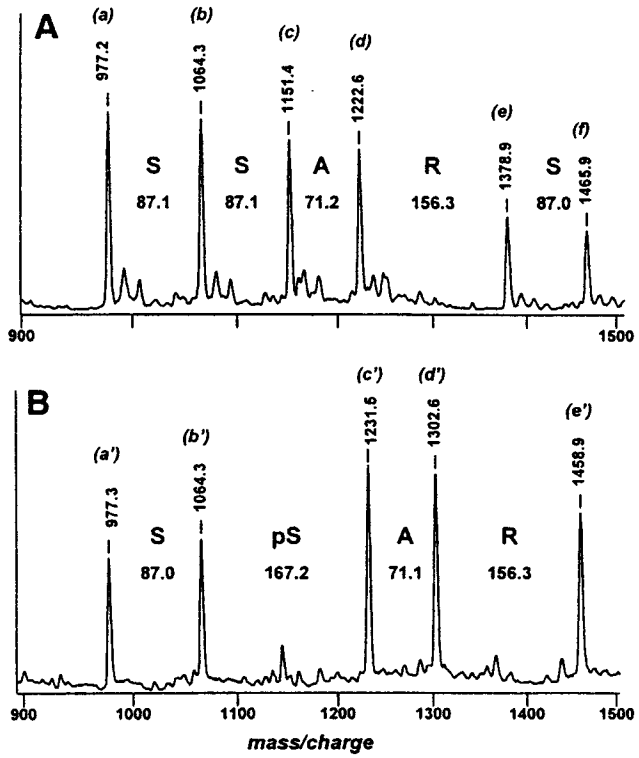

Fig. 2. Partial protein ladder-sequencing data from a peptide that is a substrate for protein kinase C. **(A)** Peptide without phosphorylation and **(B)** peptide with phosphorylation. The amino acid identities and modifications are determined by measuring the mass differences between consecutive peaks (e.g., [a] and [b], [b] and [c]) and comparing these mass differences with the amino acid residue masses (Table 3) and modified amino acid residue masses (Table 4). The amino acid sequence is determined by the order of residues from the high-mass to low-mass end of the spectrum (e.g., ... SRASS ... for spectrum A and ... RApSS ... for spectrum B). The mass shift caused by the modification is seen by comparing corresponding peaks in (A) and (B) (b with b', c with c', ...).

ferences between the other peaks in the spectrum demonstrate that no other modifications are present in this portion of the sequence.

The modified amino acid residues can be identified with special facility if both modified and unmodified peptides are available and can be subjected to ladder sequencing. As a result of the modification, the molecular masses of all of the ladder peptides that contain the modified amino acid residue will shift by an amount corresponding to the modification. This shift can readily be detected

Table 3
Masses and Compositions
of the 20 Commonly Occurring Amino Acid Residues

Average mass	Name	Three-letter code	One-letter code	Composition
57.05	Glycine	Gly	G	C_2H_3NO
71.08	Alanine	Ala	A	C_3H_5NO
87.08	Serine	Ser	S	$C_3H_5NO_2$
97.12	Proline	Pro	P	C_5H_7NO
99.13	Valine	Val	V	C_5H_9NO
101.10	Threonine	Thr	T	$C_4H_7NO_2$
103.14	Cysteine	Cys	C	C_3H_5NOS
113.16	Isoleucine	Ile	I	$C_6H_{11}NO$
113.16	Leucine	Leu	L	$C_6H_{11}NO$
114.10	Asparagine	Asn	N	$C_4H_6N_2O_2$
115.09	Aspartic acid	Asp	D	$C_4H_5NO_3$
128.13	Glutamine	Gln	Q	$C_5H_8N_2O_2$
128.17	Lysine	Lys	K	$C_6H_{12}N_2O$
129.12	Glutamic acid	Glu	E	$C_5H_7NO_3$
131.19	Methionine	Met	M	C_5H_9NOS
137.14	Histidine	His	H	$C_6H_7N_3O$
147.18	Phenylalanine	Phe	F	C_9H_9NO
156.19	Arginine	Arg	R	$C_6H_{12}N_4O$
163.18	Tyrosine	Tyr	Y	$C_9H_9NO_2$
186.21	Tryptophan	Trp	W	$C_{11}H_{10}N_2O$

by comparing the spectra of the modified and unmodified peptides (compare Fig. 2A,B).

4. Notes

1. A manual method for carrying out the ladder-generating chemistry is described in detail elsewhere *(4)*.
2. The TOF mass spectrometer is a custom linear instrument with a 2-m flight path. Peptide samples are loaded onto a multiple-sample probe containing 10 sample positions and then irradiated with pulses of laser light (wavelength 355 nm, pulse duration 10 ns) from an Nd-YAG laser source (HY 400, Lumonics, Inc., Kanata, Ontario, Canada). Ions desorbed from the probe are accelerated in two stages to an energy of 30 keV. An electrostatic particle guide is used in the flight tube to improve the ion transmission. The ions are detected by a hybrid microchannel plate/gridded discrete-dynode electron multiplier detector. The output is amplified by a fast preamplifier (Model 9305, EG&G Ortec, Oak Ridge, TN) and fed into a LeCroy Model 7200A digital oscilloscope (LeCroy, Chestnut Ridge, NY).

Table 4
Residue Masses of Some Chemically and Posttranslationally Modified Amino Acids (*see* Note 16)

Modification	Amino acid	Modified amino acid	Mass shift[a]	Modified amino acid residue mass[b]
O-linked glycosylation[c]	Ser	Dehydroalanine (Dha)	-18	69.06
O-linked glycosylation[c]	Thr	Dehydroamino-2-butyric acid (Dhb)	-18	83.09
Hydroxylation	Pro	γ-Hydroxy-Pro (Hyp)	16	113.12
Hydroxylation		β-Hydroxy-Pro	16	113.12
Hydroxylation	Asn	β-Hydroxy-Asn (βHyn)	16	130.10
Hydroxylation	Asp	β-Hydroxy-Asp (βHya)	16	131.09
Hydroxylation	Lys	δ-Hydroxy-Lys (Hyl)	16	144.17
Oxidation	Met	Met-Sulfoxide	16	147.19
Oxidation	Cys	Cysteic acid	16	151.14
Carboxylation	Asp	β-Carboxy-Asp	44	159.09
Phosphorylation	Ser	Phosphoryl-Ser	80	167.06
Carboxylation	Glu	γ-Carboxy-Glu (Gla)	44	173.12
Phosphorylation	Thr	Phosphoryl-Thr	80	181.08
Phosphorylation	Asp	Phosphoryl-Asp	80	195.07
Phosphorylation	Lys	Phosphoryl-Lys	96	208.15
Phosphorylation	His	Phosphoryl-His	96	217.12
Phosphorylation	Tyr	Phosphoryl-Tyr	80	243.16
Sulfation	Tyr	Tyrosine sulfate	80	243.24
O-linked *N*-acetylglucosamine	Ser	GlcNAc-Ser	203	290.27
O-linked *N*-acetylglucosamine	Thr	GlcNAc-Thr	203	304.30
Prenylation	Cys	Farnesyl-Cys	204	307.50
N-linked glycosylation[d]	Asn	N-GlcNAc-Asn	203	317.29
Palmitoylation	Cys	Palmitoyl-Cys	238	341.56
Prenylation		Geranylgeranyl-Cys	272	375.62
N-linked glycosylation[d]	Asn	N-Fuc-GlcNAc-Asn	349	463.44

[a]Nominal mass.

[b]The mass shifts and average masses are calculated using the atomic weights of the elements: C = 12.011, H = 1.00794, N = 14.00674, O = 15.9994, P = 30.97376, S = 32.066.

[c]β-Elimination caused by deglycosylation by dilute alkali solution (nonreductive medium) in mild conditions.

[d]Released by endo-H digestion (from high-mannose type or hybrid type *N*-linked glycosylations) or endo-F digestion of *N*-linked glycosylation.

Commercial MALDI-TOF mass spectrometers should also be suitable for reading out the masses of the ladder peptides.

3. The reagents and solvents used for mass spectrometric sample preparation should be of the highest quality available. Water is obtained by purifying distilled water through a Milli-Q UV Plus water system.
4. The molecular mass measurement helps to determine the appropriate number of sequencing cycles for the analysis. Ions originating from the matrix produce an intense background in the mass spectrum below *m/z* 500. Thus, for example, if the molecular mass of the peptide to be sequenced is ~1000 Da, the maximum number of residues that can readily be sequenced is ~5. The molecular mass is also helpful in the interpretation of the sequence data. For example, the number of lysine residues can be determined by the mass shift, since each lysine residue will react with one molecule of phenyl isothiocyanate and cause a mass shift of 135 Da. In addition to the determination of molecular mass, the measurement provides useful information regarding the purity of the peptide sample.
5. The molecular mass of the peptide of interest can be measured with other types of mass spectrometer (e.g., electrospray ionization quadrupole mass spectrometer).
6. When sufficient peptide sample is available, a 10-μ*M* solution is prepared with 0.1% TFA in water/acetonitrile (2:1 [v/v]). One microliter of peptide solution is mixed with 9 μL of matrix solution to give a final peptide concentration of 1 μ*M*. When very limited amounts of peptide are available, the peptide solution can be mixed with the matrix solution directly on the probe. In this case, 1 μL of peptide solution is rapidly added to 1 μL of matrix solution on the probe. Care must be taken to avoid precipitation of the matrix prior to addition of the peptide solution.
7. For dilute samples, the peptide solution can be applied to the membrane in multiple loadings.
8. The amount of TFA delivered should be tuned very carefully. Excessive amounts of TFA will cause peptide sample loss from the Immobilon-CD membrane.
9. High temperature during the cleavage reaction will cause dehydration side reactions as well as the oxidation of methionine residues.
10. With a cooling device, the total cycle time can be further reduced to <10 min. Steps 21, 22, and 37 (Table 1) are used to achieve the temperature changes.
11. Sonication for an extended time period can produce unwanted side reactions.
12. If necessary, the peptide solution can be concentrated using a SpeedVac (Savant Instrument Inc., Farmingdale, NY). To avoid sample loss, the solution should not allowed to dry completely.
13. The response of MALDI-MS depends on the amino acid composition of the ladder peptides. In particular, the sensitivity is enhanced when the peptide contains basic amino acid residues. Conversely, the sensitivity is reduced when no basic amino acid residues are present. In general, the order of sensitivity is Arg-containing peptides > Lys-containing peptides > peptides that do not contain basic groups, except the free N-terminal amino group > peptides containing no basic functionality. Thus, the modification on ε-amino groups of lysine residues by phenyl isothiocyanate may decrease the mass spectrometric response. This may become a serious problem for peptides that do not contain arginine residues.

14. Formally, the measured value is a mass-to-charge ratio. However, under the conditions used here, the charge is unity.
15. Leucine and isoleucine have identical residue masses (113 Da). These isobaric amino acid residues cannot be distinguished by the present ladder sequencing method. Glutamine and lysine have the same nominal residue masses (128 Da). However, they can be distinguished since the ε-amino group of the lysyl residue is modified by PITC, resulting in a mass shift of 135 (residue mass of PTC-lysine = 263.35 Da).
16. Detection of a posttranslational modification requires that the modification is not labile to the ladder-sequencing chemistry. Ambiguous results may result from posttranslationally modified amino acid residues that have similar masses to natural amino acid residues.

References

1. Chait, B. T., Wang, R., Beavis, R. C., and Kent, S. B. H. (1993) Protein ladder sequencing. *Science* **262,** 89–92.
2. Hillenkamp, F., Karas, M., Beavis, R. C., and Chait, B. T. (1991) Matrix-assisted laser desorption/ionization mass spectrometry of biopolymers. *Anal. Chem.* **63,** 1193A–1203A.
3. Wang, R., Chait, B. T., and Kent, S. B. H. (1994) Protein ladder sequencing: towards automation, in *Techniques in Protein Chemistry V* (Crabb, J. W., ed.), Academic, San Diego, CA, pp. 19–26.
4. Wang, R. and Chait, B. T. (1996) N-terminal protein ladder sequencing, in *Methods in Molecular Biology, Vol. 64: Protein Sequencing Protocols* (Smith, B. J., ed.), Humana Press, Totowa, NJ, in press.
5. Bartlet-Jones, M., Jeffery, W. A., Hansen, H. F., and Pappin, D. J. C. (1994) Peptide ladder sequencing by mass spectrometry using a novel, volatile degradation reagent. *Rapid Commun. Mass Spectrom.* **8,** 737–742.
6. Beavis, R. C. and Chait, B. T. (1989) Factors affecting the ultraviolet laser desorption of proteins. *Rapid Commun. Mass Spectrom.* **3,** 233–237.
7. Beavis, R. C., Chaudhary, T., and Chait, B. T. (1992) α-Cyano-4-hydroxycinnamic acid as a matrix for matrix-assisted laser desorption mass spectrometry. *Organic Mass Spectrom.* **27,** 156–158.