RESEARCH COMMUNICATIONS

A Method for Peptide Successive C-Terminal Degradation Using Dilute Hydrochloric Acid

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Dilute hydrochloric acid (10% w/v, 2.74 N) was reacted with peptides and proteins at 25°C for 14 days and 30 days or at 50°C for 1 to 16 hours. These reactions caused successive C-terminal degradation and deamidation of C-terminal α-amide and acidic amino acid amides. Under these conditions, the reaction also partially cleaved acid labile peptide bonds, including the C side of aspartic acid and both sides of glycine. (J Biomol Tech 1999;10:194–198)

KEY WORDS: acid hydrolysis, peptides, C-terminal sequencing, matrix-assisted laser desorption and ionization/time of flight mass spectrometry (MALDI/TOF-MS).

Protein N-terminal microsequencing protocols have been extensively developed,[1] and 100-fmol sequencing for 20 steps is now practical. The use of acid in protein chemistry was introduced by Partridge and Davis, who employed dilute hydrochloric acid (HCl) for specific weak acid peptide bond cleavage, especially at Asp and Asn residues.[2] Another use was highly concentrated HCl at room temperature by Sanger and Thompson,[3] which was specific for the N-side peptide bond cleavages of Ser and Thr, possibly by means of an N-O shift.

Double-distilled (5.7 N) HCl has been widely used for complete hydrolysis of proteins.[4] Hydrolysis efficiency is enhanced by the addition of perfluoric acid,[5] and a chemical C-terminal successive degradation method using an aqueous vapor from concentrated perfluoric acid was proposed on the basis of this enhancement.[6] This method was predicted to produce a C-terminal oxazolone reaction intermediate, which led to the use of a vapor of perfluoric anhydride to provide another successive degradation method[7] and the development of a novel stepwise C-terminal sequence procedure.[8]
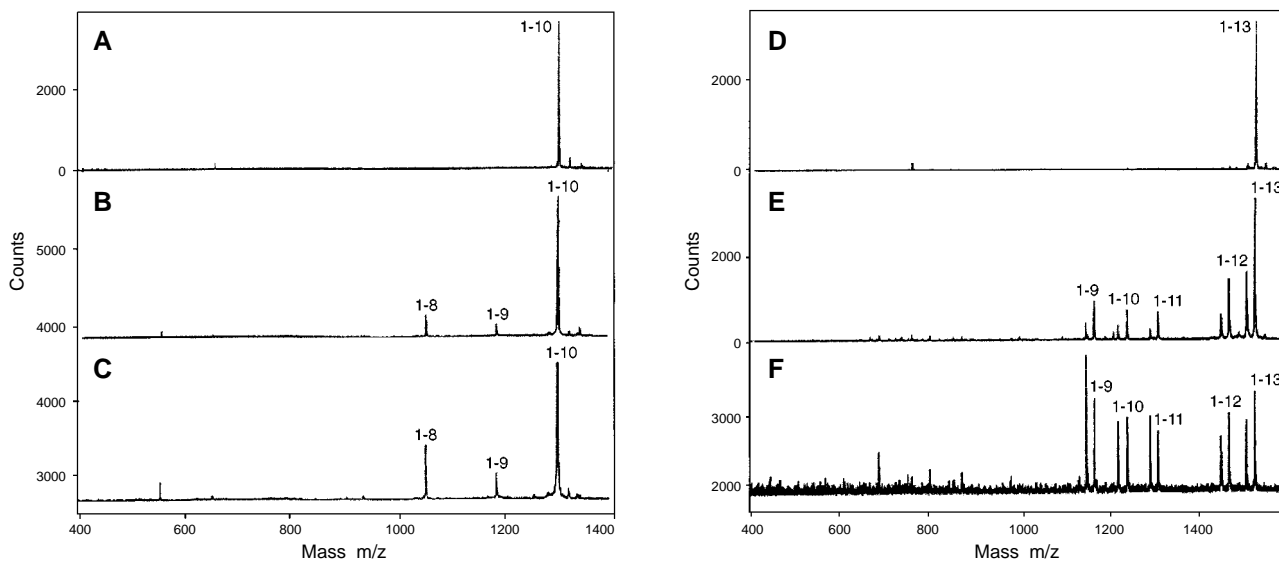
The original perfluoric acid vapor method was found to be accompanied by internal specific peptide bond cleavage, especially at the C side of Asp residues and at the N side of Ser residues in proteins.[9,10] Combination of the successive degradation and simultaneous internal specific cleavages allowed C-terminal sequencing at multiple sites of protein by using mass spectrometry (MS).[11]

Successive C-terminal degradation has been classically carried out with exoproteases such as carboxypeptidases and has been used in practice despite the disadvantages of endoproteinase contamination and insensitivity of the method. Biemann[12] has successfully introduced a tandem mass spectrometric degradation procedure for providing the N-terminal sequential fragment ions x, y, and z, as well as the C-terminal ions a, b, and c. Peptide C-terminal sequencing by MS has been extensively studied, and different types of ionization-based MS instruments have been developed that allow higher sensitivity and an extended range of molecular size. matrix-assisted laser desorption and ionization/time of flight (MALDI/TOF) MS with a high-energy laser beam has been employed for this pupose.[13]

This report describes another novel chemical C-terminal successive degradation method that may be easily carried out, has a reasonably high sensitivity (picomole level), and has an analyzable molecular size of 20 kd because of the mass spectrometer used. The extent of the C-terminal sequence determination is, however, limited by the presence of several amino acid residues.

MATERIALS AND METHODS

Substance P, an undecapeptide (RPKPQQFFGLM-NH$_2$, M$_r$ 1347); bombesin, a dodecapeptide pyrrolidone–carboxylic acid (pGlu-QRLGNQWAGHLM-NH$_2$, M$_r$ 1619); angiotensin I, a decapeptide (DRVYIHPFHL); a tyrosine protein kinase phosphorylation substrate, a tridecapeptide (RRLIEDAEY AARG, M$_r$ 1519); and human glucagon (HSQGTFTSDYSK

FIGURE 1

MALDI-TOF mass spectra of 10% HCl reactions for two peptides (each 5 pmol) at room temperature (25°C). Angiotensin I (DRVYIHPFHL): **(A)** 0 time, **(B)** 14 days, and **(C)** 30 days. Tyrosine protein kinase substrate (RRLIEDAYARG): **(D)** 0 time, **(E)** 14 days, and **(F)** 30 days; four amino acid residues were successively liberated from the C terminus with accompanied the −18 u peaks.

YLDSRRAQDFVQWLMNT, M_r 3482.7) were obtained from Peptide Institute, Inc. (Minoh, Osaka, Japan). Dilute HCl (10% w/v, 2.74 N) and 0.1 N HCl were obtained from Wako Pure Chemical Industry (Osaka, Osaka, Japan).

Peptide or protein (5 pmol) was dissolved in 4 μL of 10% HCl and sealed in a 10-μL capillary glass tube under argon. The tube was incubated for a specified period and a specified temperature. The tube was opened, and 1 μL was used for mass spectrometric analysis.

MALDI MS was carried out on a Voyager-DE-STR time of flight (TOF) mass spectrometer (Perspective Biosystem, MA, USA) equipped with a model VSL-337ND nitrogen laser (Laser Science, MA, USA; 337 nm, 3 ns pulse length) and a dual microchannel plate detector (Galileo, MA, USA). The acceleration voltage in the ion source was 20 kV. Linear mode and delayed extraction conditions were employed for these experiments. Reacted sample solution in 10% HCl (1 μL) was mixed with 4 μL of a matrix that consisted of a mixture of α-cyano-4-hydroxy cinnamic acid (1 mg) and 100 μL of 0.1% trifluoroacetic acid/acetonitrile (3:2 v/v). A portion (0.5 μL) of the sample and matrix solution was deposited on the sample plate, and the solvents were then removed by drying the sample in air at room temperature.
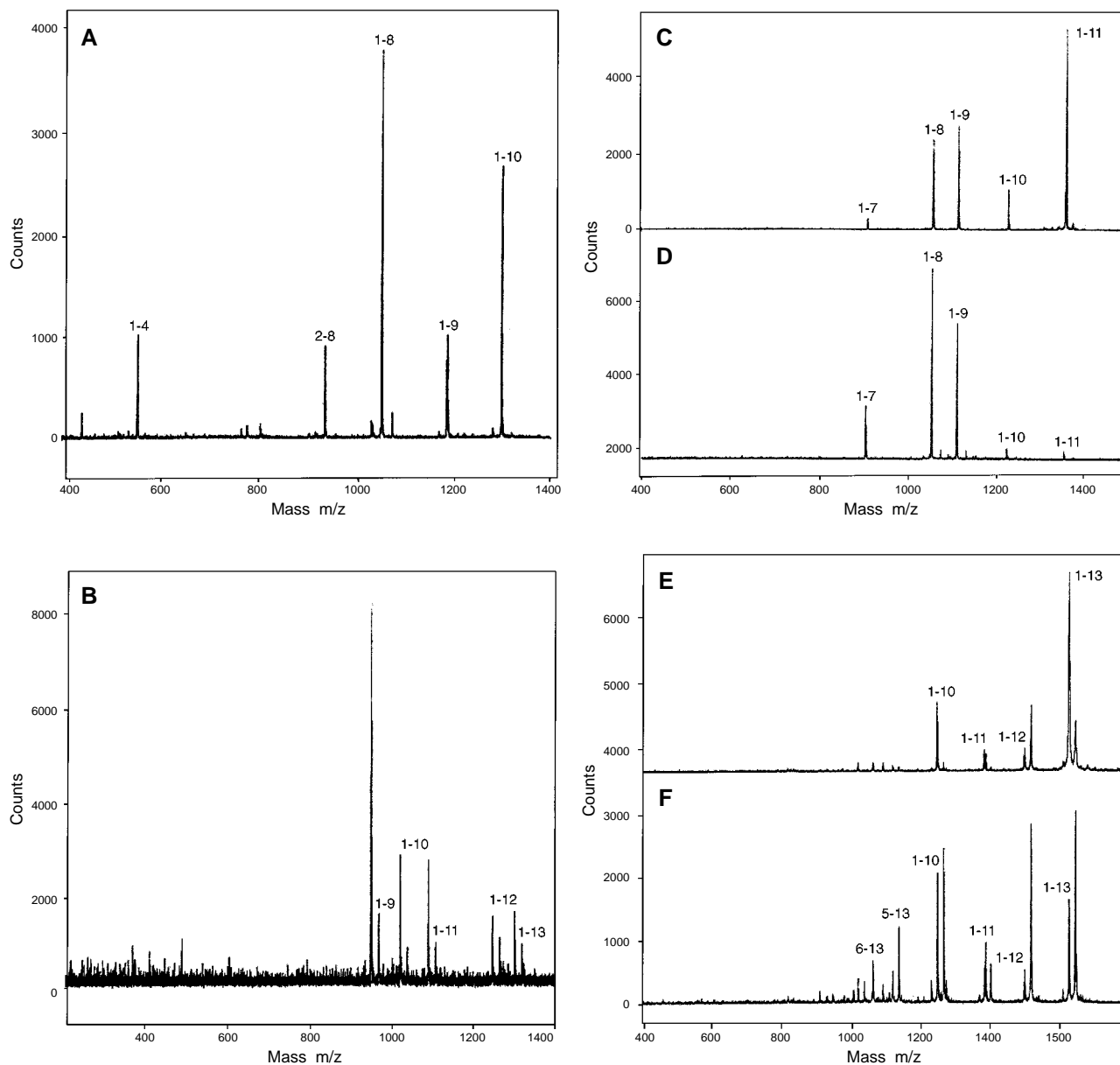
RESULTS AND DISCUSSION

When we happened to leave a decapeptide, angiotensin I (DRVYIHPFHL, M_r 1296), at room temperature (25°C) for 2 months in 10% HCl aqueous solution, we observed by MALDI/TOF-MS analysis evidence of C-terminal successive degradation of the peptide. Further experiments were then carefully conducted to confirm this observation by standing the same peptide in 10% HCl for 4 days, 10 days, 14 days,

30 days, and 36 days at 25°C. Some selected data are shown in Figure 1 for 0 time, 14 days, and 30 days. The maximum degradation of the peptide was achieved at 30 days. We observed successive liberation of two amino acids from the C terminus and then no further amino acid release nor modification of the constituent amino acid residues. We questioned whether this observation was specific for this particular peptide. Tridecapeptide, a tyrosine protein kinase substrate (RRLIEDAEYAARG, M_r 1519), was left for 30 days at 25°C. As shown in Figure 1D–F, four amino acid residues were successively liberated from the C terminus together with accompanying −18 u peaks. These −18 u peaks may result from dehydration of some N-terminal residue (1-8) in the peptide or possible cyclization of the Asp^6 or Glu^5 residue.

In an effort to shorten the reaction time, several peptides, including angiotensin I and tyrosine protein kinase substrate, were treated with HCl solution at 50°C for up to 16 hours. An undecapeptide, substance P (RPKPQQFFGLM-NH_2, M_r 1347), reacted at 50°C for 4 and 16 hours, and a dodecapeptide, bombesin (pGlu-QRLGNQWAGHLM-NH_2, M_r 1619), reacted for 1 and 4 hours, were also tested.

The results of MALDI/TOF-MS analyses of the products are summarized in Figure 2. For all peptides, two to five amino acids were liberated, all from C termini. However, the high temperature of 50°C apparently modified some constituent amino acid residues, including deamidation (+1 u) of C-terminal α-carboxyl amides, as well as amides of Gln and Asn. Hydration of the pyrrolidone N terminus to Glu (+18 u) in bombesin and internal peptide bond cleavages such as a partial one at Asp^1-Arg^2 in angiotensin I were also observed. Neither Asp^6-Ala^7 peptide bond cleavage in

FIGURE 2

MALDI-TOF mass spectra of 10% HCl reactions for four peptides (each 5 pmol) at 50°C. Angiotensin I (DRV YIHPFHL): **(A)** 16 hours. Tyrosine protein kinase substrate (RRLIEDAEYAARG): **(B)** 16 hours. Substance P (RPKPQQFFGLM-NH$_2$): **(C)** 1 hour and **(D)** 16 hours. Bombesin (pGlu-QRLGNQWAGHLM-NH$_2$): **(E)** 1 hour and **(F)** 4 hours. The data show dehydration of N-terminal pyrrolidon-carboxylic acid (+18 u) and deamidation of α-carboxyl amide, Asn, and two Gln residues (+4 u).

tyrosine protein kinase substrate nor oxidation of Trp and Met residues were observed. However, dehydration was observed for a Glu-containing peptide, the tyrosine protein kinase substrate. At 50°C, angiotensin I gave two series of truncation peaks (Fig. 2*A*), including those produced by acid-specific cleavage of the Asp^1-Arg^2 peptide bond (which resulted in [M+H]$^+$ at the m/z 932 peak in Fig. 2*A*). The C-terminal degrading reaction did not progress further, perhaps because of an inability to degrade beyond a prolyl-X

bond, where X is any amino acid. The tyrosine protein kinase substrate exposed to 50°C for 16 hours resulted in the spectrum shown in Figure 2*B*. This revealed removal of four amino acids from the C terminus, with each truncation peak accompanied dehydration peaks, possibly derived from cyclization of an Asp^6 or Glu^5 residue. Degradation stopped at the Tyr^9 residue.

When substance P was subjected to 50°C for 1 hour (Fig. 2*C*) and for 16 hours (Fig. 2*D*), it was observed that the
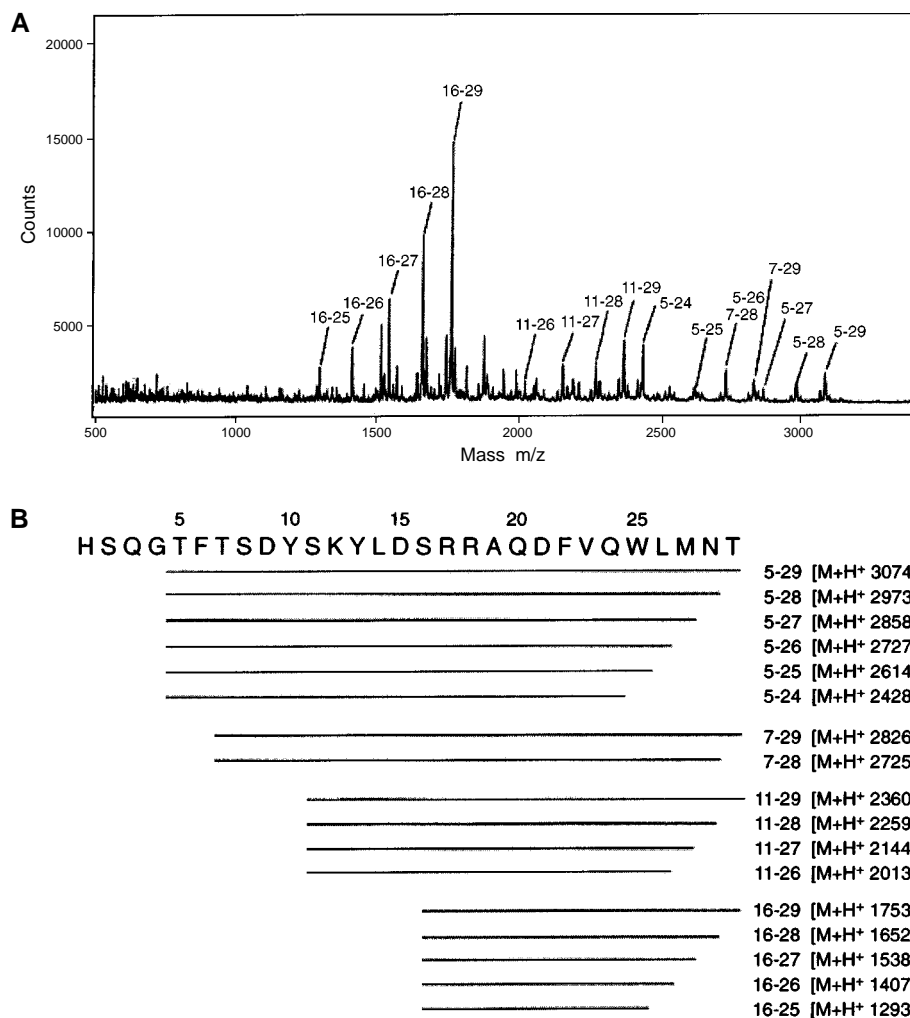
A

B

FIGURE 3

(A) MALDI/TOF mass spectrum of a 10% HCl reaction with human glucagon (5 pmol) at 50°C for 16 hours. **(B)** The cleavage and molecular ions are schematically illustrated.

α-carboxyl amide was hydrolyzed, followed by truncation of three amino acids from the C terminus. After 1 hour, the C-terminal two amino acids were liberated almost quantitatively, whereas Phe[8] was only partially cleaved. When the cleavage was extended to a 16-hour reaction, Phe[8] was resistant to further reaction. The two Gln[5] and Gln[6] residues were deamidated. In contrast, bombesin resulted in truncation after a 1-hour reaction (Fig. 2E) and 4-hour reaction (Fig. 2F). The data showed hydration of the N-terminal pyrrolidon-carboxylic acid (+18 u) and deamidations of the α-carboxyl amide, Asn[6], and the two Gln[2] and Gln[7] residues (+4 u). For this peptide, the presence of the Trp[8] residue prevented further reaction.

The large polypeptide or small protein, glucagon, was then subjected to 10% HCl at 50°C for a 16-hour reaction. We observed cleavage of internal peptide bonds and C-terminal truncation from these cleavage sites. Figure 3 presents the MALDI/TOF MS and the schematically presented cleavage and truncated molecular ions. The reaction was stopped by the aromatic amino acid residue, Trp[25].

This method is similar to the perfluoric acid C-terminal truncation reaction,[6] although the two differ at certain points. This method decomposes C-terminal and internal amides, whereas the perfluoric acid reaction does not. However, this difference is potentially useful when studying C-terminal amidated peptides. In comparison, 0.1 M HCl did not give clear C-terminal truncation but only deamidation (data not shown). Continuation of the truncation reaction is inhibited at the Pro-X peptide bond in both truncation reactions and slowed or stopped at aromatic amino acids in the current truncation.

In summary, when peptide or protein was reacted with 10% HCl at 50°C for 1 to 16 hours, successive C-terminal degradation was observed. This reaction stopped at Pro-X peptide bonds and in most cases at aromatic amino acid residues. The reaction also deamidated the C-terminal α-amide and the amides of Asn and Gln. The order of reactivities on amides was indistinguishable. Internal peptide bond cleavages were also observed at the C-terminal side of Asp, N-terminal sides of Ser and Thr, and both sides of Gly.

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