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Bond-Specific Chemical Cleavages of Peptides and Proteins with Perfluoric Acid Vapors: Novel Peptide Bond Cleavages of Glycyl-Threonine, the Amino Side of Serine Residues and the Carboxyl Side of Aspartic Acid Residues

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Peptide bond cleavages by vapors composed of various from aqueous solutions of perfluoric acid were studied using synthetic peptides and proteins, and specific conditions were established for peptide bond cleavages including a novel cleavage of the glycyl-threonine bond. The peptide bonds on the aminosides of serine residues were cleaved by exposure to a vapor of 75% aqueous heptafluorobutyric acid at 30 or 50°C for 24 h. Glycyl-threonine peptide bonds were cleaved with vapors of various concentrations (5, 75, and 90%) of heptafluorobutyric acid at 30-40°C for 24 h. The peptide bonds on the carboxylsides of aspartic acid residues were cleaved by exposure to a vapor of 0.2% heptafluorobutyric acid at 90°C for 4 to 24 h. The same vapor cleaved aspartyl-proline bonds under milder conditions such as at 60°C for 16 h, under which the other aspartyl bonds were uncleaved. These specific chemical cleavages were applied to several proteins including newly characterized proteins.

Key words: Asp-C cleavage, Asp-Pro cleavage, Gly-Thr cleavage, perfluoric acid, Ser-N cleavage.

In the previous paper (*1*) we reported a method for successive degradation of peptides from the carboxyl (C) terminus with a vapor of 90% aqueous perfluoric acid at 90°C between 4 and 24 h, resulting in the C-terminally truncated molecules which were then analyzed by fast atom bombardment (FAB) or electrospray-ionization mass spectrometry. Two types of side reactions of internal peptide bond cleavages were observed during the C-terminal truncation, *i.e.*, at the amino (N)-side of serine residues and the C-side of aspartic acid residues.

Sanger and Thompson (*2*) reported selective peptide bond cleavages for peptides with serine, threonine and glycine residues in concentrated hydrochloric acid at room temperature. The reaction mechanism of the cleavages at the N-side of both serine and threonine was explained by the N→O shift of hydroxyl groups.

Partridge and Davis (*3*) observed that aspartic acid was the only amino acid released when proteins were heated in weak acid solutions. This cleavage was specific for aspartic acid, but later it was found to be accompanied by the hydrolysis of amide groups of asparagine residues. Inglis (*4*) recently studied these cleavage reactions under controlled conditions, such as pH 2 at 108°C for 2 h, which generated both N- and C-side cleavages of aspartic acid residues. He also reported the conditions under which selective cleavage at the C-side of aspartic acid was obtained.

Specific cleavage of aspartyl-proline bonds has been commonly used since Poulsen *et al.* (*5*) reported the cleavage conditions of rabbit antibody light chain. Landon (*6*) summarized the conditions suitable for this type of cleavage.

In the present study we report a series of experiments which establish the conditions for the bond-specific cleavages of a novel Gly-Thr peptide bond, serine N-side peptide bonds, aspartic acid C-side bonds, and aspartyl-proline bonds in vapor phases generated from aqueous heptafluorobutyric acid ($C_3F_7CO_2H$).

MATERIALS AND METHODS

Materials—$C_3F_7CO_2H$ was purchased from Sigma Chemical Company (USA) and dithiothreitol (DTT) was obtained from Nacalai Tesque (Kyoto). Human vasoactive intestinal peptide precursor fragment 111-122, Val-Ser-Ser-Asn-Ile-Ser-Glu-Asp-Pro-Val-Pro-Val, was the synthesized product of Bachem Feinchemikalien AG (Switzerland). Dipeptides, Gly-Thr and Gly-Ser, were obtained from Kokusan Chemical Works (Tokyo). Human parathyroid hormone fragment 69-84, Glu-Ala-Asp-Lys-Ala-Asp-

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Abbreviation: $C_3F_7CO_2H$, heptafluorobutyric acid; CGMMV, cucumber green mottle mosaic virus; FAB, fast atom bombardment; PVDF, polyvinylidene difluoride; TMV, tobacco mosaic virus.

Val-Asn-Val-Leu-Thr-Lys-Ala-Lys-Ser-Gln, and the other peptides used in the experiments were the products of Peptide Institute (Minoh). Calf thymus histone H2A was obtained from Boehringer Mannheim (Germany). It was further purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) (*7*) and extracted from the gel with 70% formic acid followed by Bio-Gel® P-10 (Bio-Rad Laboratories, USA) molecular sieving (*8*). Coat proteins from cucumber green mottle mosaic virus (CGMMV) and tobacco mosaic virus (TMV) were donated by Dr. Yuzo Nozu (National Institute of Agrobiological Resources, Tsukuba). Myoglobin of Japanese cormorant (*Phalacrocorax filamentosus*) was purified by the conventional method, 85% ammonium sulphate precipitation. TM-1 protein from the venom of Taiwan habu (*Trimeresurus mucrosquamatus*) was donated by Prof. S.-H. Chiou (Institute of Biological Chemistry, National Taiwan University, Taipei, Taiwan). The sequence analysis of myoglobin and TM-1 is in progress in our laboratory. Horse heart cytochrome *c* was obtained from Serva Feinbiochemica GmbH & Co. (Germany), and other proteins were from Sigma. Polyvinylidene difluoride (PVDF) membrane (Immobilon™-P^{SQ}) was the product of Millipore Corporation (USA).

Acid Cleavage of Peptide or Protein—Acid vapor cleavage reactions were basically performed according to the procedure in the previous paper (*1*). Proteins or peptides were dried in a small glass tube (6 mm × 40 mm). The tube was placed in a larger glass tube (13 mm × 100 mm), which contained 100 μl of aqueous $C_3F_7CO_2H$ of a given concentration between 0.2 and 90%, containing 5% (mass/volume) DTT. The double tube was cooled in 1-propanol and dry-ice (−70°C) and flame-sealed under vacuum (1 Pa). The tube was placed in a water bath at a specified temperature between 30 and 90°C for 4 to 24 h. After the reaction, the tube was opened and the small tube was transferred into a vacuum desiccator to remove traces of acid.

Fast Atom Bombardment (FAB) Mass Spectrometry—The reaction mixture was dissolved in 2 μl of 67% acetic acid and mixed with 1 μl of matrix consisting of glycerol, thioglycerol, and *m*-nitrobenzylalcohol (1 : 1 : 1, by volume). FAB mass spectrometry was carried out with a HX-110 mass spectrometer (JEOL, Tokyo), equipped with a DA5000 data system, employing an accelerating voltage of 10 kV and xenon as an ionizing gas.

SDS/PAGE—The product of the acid-cleaved protein was dissolved in the PAGE sample buffer (*7*). The solution was boiled for 5 min and subjected to SDS/Tricine/PAGE (*9*). To detect the cleaved fragments, the gel was stained with silver or Coomassie Brilliant Blue R-250.

N-Terminal Sequence Analysis—After electrophoresis, the peptide bands on the SDS-gel were electroblotted onto a PVDF membrane according to Ploug *et al.* (*10*) with a semi-dry blotting system (Nihon-Eido, Tokyo) at 0.5 mA/cm^2 for 2 h. The membrane was stained with 0.1% Coomassie Brilliant Blue, and the stained band was excised and transferred to a protein sequencer (model 477A, Perkin Elmer Corporation, USA). The sequencing was carried out according to the sequencer manual, and phenylthiohydantoin (PTH)-amino acids were identified with an on-line HPLC system (model 120A, Perkin Elmer) (*11*).

Amino Acid Analysis—Amino acid analysis was performed with an amino acid analyzer model A8700 (Irica Instrument, Kyoto) at a sensitivity setting of 2.5 nmol full scale. The analyzer was equipped with cation-exchange chromatography and a standard ninhydrin detection system.

RESULTS AND DISCUSSION

In the previous study (*1*) we chose vapors from either aqueous pentafluoropropionic acid ($C_2F_5CO_2H$) or $C_3F_7CO_2H$ for C-terminal truncation but not from trifluoroacetic acid (CF_3CO_2H), which was observed to produce undesired by-products. DTT (5%) was always added to the acid solutions to prevent oxidation or oxidative modifications of peptide and protein. The reaction was carried out with 90% of aqueous $C_3F_7CO_2H$ (or $C_2F_5CO_2H$) in the vapor phase at 90°C. The C-terminally truncated molecules were observed to be accompanied by internal peptide bond cleavages.

Preliminary Studies on Internal Acid Cleavage Reactions—To further characterize the internal peptide cleavages, several peptides were exposed to vapors from various concentrations of aqueous $C_3F_7CO_2H$ solution between 0.01 and 90% at either 50 or 90°C for 24 h, and the products were analyzed by FAB mass spectrometry. The C-terminal truncation was observed at 90°C with vapors of aqueous $C_3F_7CO_2H$ higher than 70%, as reported in the previous paper (*1*), but little cleavage was observed at temperatures lower than 50°C with vapors from any acid concentration. However, the internal peptide bond cleavages on the N-side of serine and the C-side of aspartic acid were observed under milder conditions.

The extent of cleavage by vapors from various aqueous $C_3F_7CO_2H$ concentrations on a hexadecapeptide, Glu-Ala-Asp-Lys-Ala-Asp-Val-Asn-Val-Leu-Thr-Lys-Ala-Lys-Ser-Gln, at both 50°C (Fig. 1A) and 90°C (Fig. 1B), was analyzed by FAB mass spectrometry. At 50°C, Ser-N cleavages were observed with vapors of aqueous $C_3F_7CO_2H$ higher than 10%, while the extent of Asp-C cleavage was low even with vapor of 90% acid. At 90°C, however, Ser-N cleavages were observed with acid vapors higher than 1.0% and Asp-C cleavages were observed with acid vapors between 0.01 and 1.0%. These results indicated that the reaction conditions for the two kinds of internal cleavages were clearly distinguishable from each other and from those of the C-terminal truncation. Details of these reaction conditions are discussed in the following sections.

Serine N-Side Cleavage—In preliminary experiments, selective cleavage on the N-side of serine residues was observed at 50°C with aqueous $C_3F_7CO_2H$ vapors higher than 10%, and the yield of Ser-N cleavage decreased sharply with acid concentrations lower than 10%.

The same hexadecapeptide as in the previous section was exposed to vapors from aqueous $C_3F_7CO_2H$ of between 70 and 85% at 50°C for 24 h (Fig. 2). The reaction with vapor from 70% acid solution cleaved the peptide at the N-side of Ser-15 and one of the fragments, 1-14, was observed in the FAB mass spectrum as shown in Fig. 2A. Cleavage yields were roughly estimated from the height of molecular ion peaks. The yield was 16% with the vapor of 70% $C_3F_7CO_2H$, and increased to 27% with a vapor of 75% $C_3F_7CO_2H$, where the C-terminal truncation was almost undetectable (Fig. 2B). Cleavages on the C-sides of Asp-3 and Asp-6, in addition to the N-side of Ser-15, appeared when the peptide was exposed to vapors of acid solutions higher than 80% (Fig. 2, C and D). The yields for Ser-N were

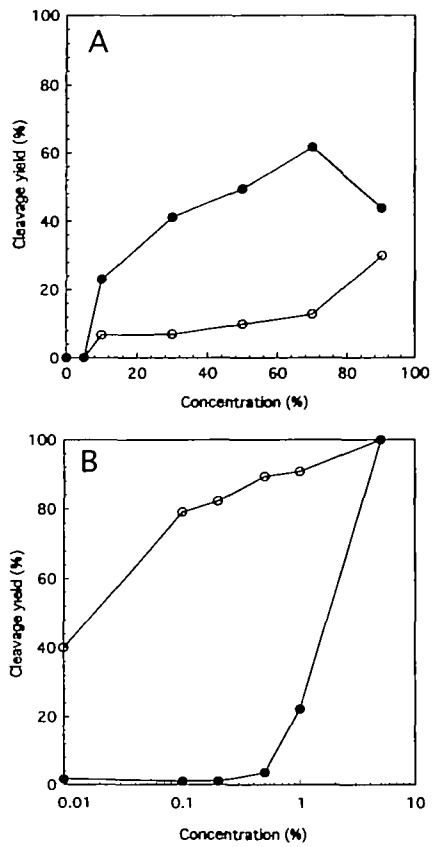

Fig. 1. **Ser-N and Asp-C cleavage reactions on a synthetic peptide.** A hexadecapeptide, Glu-Ala-Asp-Lys-Ala-Asp-Val-Asn-Val-Leu-Thr-Lys-Ala-Lys-Ser-Gln, was exposed to vapors from various concentrations of aqueous $C_3F_7CO_2H$ containing 5% DTT for 24 h at (A) 50°C and (B) 90°C. The products were analyzed by FAB mass spectrometry and cleavage yields for both Ser-N (●) and Asp-C (○) were estimated from the relative heights of molecular ions.

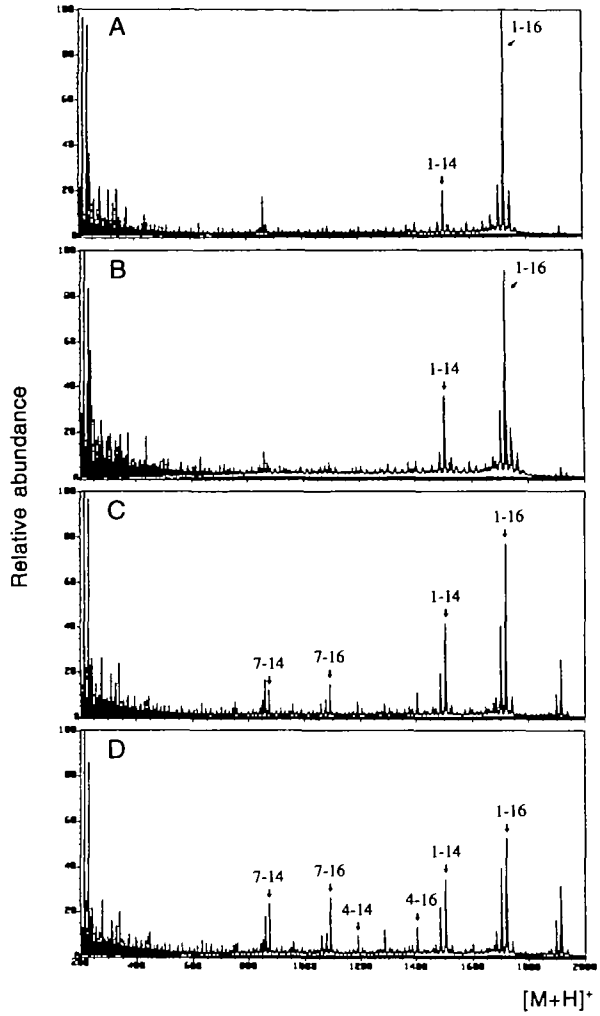

Fig. 2. **FAB mass spectra of Ser-N cleavage products of a hexadecapeptide.** A hexadecapeptide, Glu-Ala-Asp-Lys-Ala-Asp-Val-Asn-Val-Leu-Thr-Lys-Ala-Lys-Ser-Gln, was exposed to vapors from various concentrations of aqueous $C_3F_7CO_2H$ containing 5% DTT at 50°C for 24 h. The vapors were generated from (A) 70%, (B) 75%, (C) 80%, and (D) 85% aqueous $C_3F_7CO_2H$ solutions.

estimated to be 29% with 80% acid vapor and 21% with 85% acid vapor, while those for Asp-C from both Asp-3 and Asp-6 were 17% with 80% acid vapor and 45% with 85% acid vapor. Although this peptide contains the other acid-labile peptide bonds, such as those for Asn-8 and Thr-11, these peptide bonds were cleaved only marginally under these conditions, as Fig. 2 shows.

Three other peptides were exposed to vapor of 75% aqueous $C_3F_7CO_2H$ at 50°C for 24 h (Fig. 3). For a decapeptide, Lys-Arg-Pro-Hyp-Gly-Phe-Ser-Pro-Phe-Arg, the N-side cleavage of Ser-7 was clearly observed, resulting in two the peptide fragments 1-6 and 7-10, but no other cleavage was detected. The yield of cleavage was estimated to be 66% (Fig. 3A). A pentadecapeptide, Lys-Ile-Leu-Gly-Asn-Gln-Gly-Ser-Phe-Leu-Thr-Lys-Gly-Pro-Ser-Lys-Leu, having two serine residues, was observed to be cleaved mainly at the N-side of Ser-8, as shown by two molecular ions, 1-7 and 8-17 (yield 58%), but little at the N-side of Ser-15 (yield 3%), where the Pro-Ser peptide bond was resistant to the present acid vapor (Fig. 3B). The other acid-labile peptide bonds of residues Gly-4 and -13, Asn-5 and Thr-11 were not cleaved. The resistance of the Pro-Ser bond may be a general phenomenon. It was also observed for an undecapeptide, Glp-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu. Of the two peptide bonds involving serine residues, Pro-Ser and Arg-Ser, only the latter was cleaved (65%), resulting in two peptide ions, 1-7 and 8-11 (Fig. 3C).

Serine N-side cleavages were observed at 50°C with vapors from aqueous $C_3F_7CO_2H$ higher than 10% for 24 h. At 70°C with acid concentrations higher than 70%, C-terminal truncation appeared. These findings indicated that the range of acid concentration to be used for specific serine N-side cleavage is rather wide, 50-75%. However, the temperature should be kept below 50°C and the incubation time shorter than 24 h in order to avoid side reactions.

Proteins were tested for the serine N-side cleavage under the conditions suggested above and the products were separated by SDS/Tricine/PAGE as described in "MATERIALS AND METHODS." Alcohol dehydrogenase from yeast, both coat proteins from CGMMV and TMV, and TM-1 protein from snake venom were exposed to the vapor of 75% aqueous $C_3F_7CO_2H$ containing 5% DTT at 30°C for 24 h. The lower temperature of 30°C was chosen for protein cleavage to avoid the side reactions. The PAGE patterns

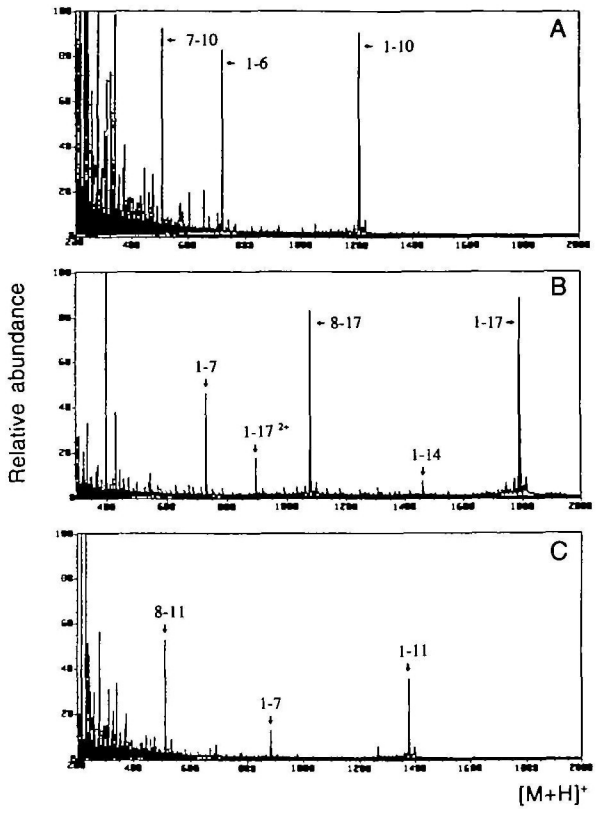

Fig. 3. **FAB mass spectra of Ser-N cleavage products of three peptides.** The reactions were performed at 50°C for 24 h with the vapor from 75% aqueous $C_3F_7CO_2H$ containing 5% DTT on (A) a decapeptide, Lys-Arg-Pro-Hyp-Gly-Phe-Ser-Pro-Phe-Arg, (B) a pentadecapeptide, Lys-Ile-Leu-Gly-Asn-Gln-Gly-Ser-Phe-Leu-Thr-Lys-Gly-Pro-Ser-Lys-Leu, and (C) an undecapeptide, Glp-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu, where Hyp and Glp stand for hydroxyproline and pyroglutamic acid, respectively.

(Fig. 4) clearly indicated specific fragmentation of these proteins. The partial N-terminal amino acid sequences of the peptide bands electroblotted onto PVDF membrane were determined. Table I lists the apparent molecular masses of peptide bands observed on SDS/PAGE, the N-terminal sequences of the bands, the residues preceding the

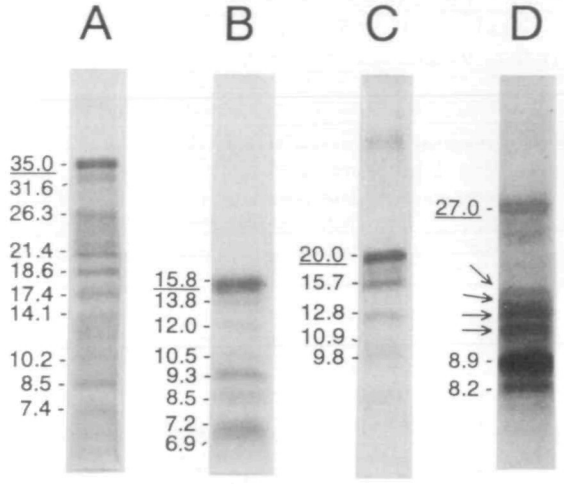

Fig. 4. **SDS/Tricine/PAGE patterns of the Ser-N cleavage products.** Proteins were treated with 75% aqueous $C_3F_7CO_2H$ vapor containing 5% DTT at 30°C for 24 h. The products were applied to SDS/Tricine/PAGE on 16.5% gel. Lane A, the cleavage product of alcohol dehydrogenase; lane B, the cleavage product of CGMMV coat protein; lane C, the cleavage product of TMV coat protein; lane D, the cleavage product of TM-1 protein. Numbers indicate apparent molecular masses (kDa) of respective bands, and the numbers with underlines indicate the intact proteins. The yields of bands in the lanes A, B, and C were approximately 10%, and that of lane D was 80% of the respective intact untreated proteins. These values were estimated both from optical densities of bands and the sequence data. The bands marked with → were observed for the intact protein preparation without acid treatment.

TABLE I. **Sequences of the Ser-N cleavage products of proteins.** Proteins were treated with 75% $C_3F_7CO_2H$ vapor containing 5% DTT at 30°C for 24 h. Apparent molecular masses correspond to those in Fig. 4. * stands for the C-terminal residue of each protein. Three bands, 31.6 and 26.3 kDa in Fig. 4A and 8.5 kDa in Fig. 4B, were observed to contain two or more fragments by the sequence analyses, and the cleavage sites could not be identified from the experimental data.

Protein (kDa)	Apparent molecular mass (kDa)	Preceding residue	N-terminal sequence	Possible C-terminal cleaved site ↓
Alcohol dehydrogenase	21.4	142-Gly	Thr-Asp-Leu-Ala-Gln-	*-347
(35.0)	18.6	183-Gly	Ser-Leu-Ala-Val-Gln-	*-347
	17.4	*N*-acetyl	Ser-Ile-Pro-Glu-Thr-	-Lys-Ser-164
	14.1	*N*-acetyl	Ser-Ile-Pro-Glu-Thr-	-Gly-Ser-124
	10.2	*N*-acetyl	Ser-Ile-Pro-Glu-Thr-	-Gly-Ser-96
	8.5	263-Gly	Thr-Thr-Val-Leu-Val-	*-347
	7.4	277-Cys	Ser-Asp-Val-Phe-Asn-	*-347
CGMMV coat protein	13.8	15-Ala	Ser-Tyr-Val-Pro-Val-	-Ala-Ser-143
(15.8)	12.0	32-Gly	Thr-Ala-Phe-Gln-Thr-	-Ala-Ser-143
	10.5	48-Leu	Ser-Ala-Leu-Pro-Ser-	-Ala-Ser-143
	9.3	59-Asn	Ser-Arg-Phe-Pro-Asp-	-Ala-Ser-143
	7.2	98-Asp	Pro-Ser-Asn-Pro-Thr-	*-160
	6.9	98-Asp	Pro-Ser-Asn-Pro-Thr-	-Thr-Ser-158
TMV coat protein	15.7	*N*-acetyl	Ser-Tyr-Ser-Ile-Thr-	-Gly-Thr-136
(20.0)	12.8	48-Phe	Ser-Glu-Val-Trp-Lys-	*-158
	10.9	64-Asp	Ser-Asp-Phe-Lys-Val-	*-158
	9.8	77-Asp	Pro-Leu-Val-Thr-Ala-	*-158
TM-1	8.9	123-Tyr	Ser-Val-Gly-Leu-Val-	*-203
(27.0)	8.2	131-His	Ser-Ser-Asn-Val-Phe-	*-203

N-termini, the residue numbers in the respective proteins, the C-terminal cleaved peptide bonds with the residue numbers predicted from the apparent molecular masses and the original protein C-terminus marked with *.

In alcohol dehydrogenase, peptide bonds 183-Gly-Ser-184 and 277-Cys-Ser-278 were cleaved, and the cleavages of 163-Lys-Ser-164, 123-Gly-Ser-124 and 95-Gly-Ser-96 were predicted from the molecular masses of the respective peptides. This protein has an N-terminal acetyl-serine. Three bands, 17.4, 14.1, and 10.2 kDa, resulted from the internal cleavages and removal of *N*-acetylgroup of the protein. The N→O shift reaction is known to be easier for an acetyl group than for an amino acid residue (or polypeptide) neighboring the N-terminal side of a Ser (or Thr) residue. The deblocking of N-terminal acetyl-serine was preliminarily reported in previous papers (*12, 13*). Altogether, 6 of 21 possible serine N-side cleavages were observed or predicted. This ratio does not reflect the real situation, because the present detection method has limitations, such as being unable to detect small molecules. An additional specific cleavage was observed for the peptide bonds 142-Gly-Thr-143 and 263-Gly-Thr-264. The N→O shift is known not only for serine but also for threonine (*2*). Although this protein has 14 threonine residues, the threonine N-side cleavages were observed only for Gly-Thr, and these two Gly-Thr bonds are the two Gly-Thr bonds cleaved being the only two such bonds among the 14 Thr-N bonds in the protein.

The cleavage of CGMMV coat protein revealed five cleavage sites, 15-Ala-Ser-16, 48-Leu-Ser-49 and 59-Asn-Ser-60, and possibly 142-Ala-Ser-143 and 157-Thr-Ser-158. This protein has 22 serine residues. Two other types of cleavage were also shown. One again involved a Gly-Thr bond, 32-Gly-Thr-33; and the other involved the 98-Asp-Pro-99 bond, which is known to be of the most acid-labile type of peptide bond as described later. The CGMMV coat protein has 12 threonine residues and an acetyl-alanine as the N-terminus, all of which are uncleaved.

TMV coat protein was cleaved at the N-terminal acetyl-Ser, 48-Phe-Ser-49 and 64-Asp-Ser-65. One Gly-Thr cleavage was observed at 135-Gly-Thr-136. This protein has 16 serine and 16 threonine residues. Of the two Asp-Pro bonds in this protein, only 77-Asp-Pro-78 was observed to be cleaved.

The TM-1 protein showed two serine N-side cleavages, 123-Tyr-Ser-124 and 131-His-Ser-132. This protein contains 13 serine residues.

The sequence data showed cleavages on the N-side of serine, including *N*-acetyl serine, and cleavage of the Asp-Pro bond. The Gly-Thr bond was also cleaved under the present conditions. Ser-Ser bond cleavage was not observed for these three proteins: CGMMV coat protein contains two Ser-Ser sequences; TMV coat protein has two Ser-Ser and one Ser-Ser-Ser; TM-1 has two Ser-Ser. No Pro-Ser bonds were cleaved: three in CGMMV coat protein and two in TMV coat protein.

Glycyl-Threonine Peptide Bond Cleavage—In the above three proteins, all Gly-Thr peptide bonds were observed to be cleaved but the Thr-N bonds were not under the conditions for the Ser-N cleavage. The uncleaved peptide bonds are as follows: two each of Ala-Thr, Asp-Thr and Ser-Thr, and one each of Glu-Thr, His-Thr, Ile-Thr, Phe-Thr, Thr-Thr, and Tyr-Thr in alcohol dehydrogenase; two each of Arg-Thr, Ser-Thr and Thr-Thr, and one each of Ala-Thr, Asp-Thr, Gln-Thr, Ile-Thr, and Pro-Thr in CGMMV coat protein; three Ala-Thr, and two each of Thr-Thr and Val-Thr, and one each of Arg-Thr, Asp-Thr, Cys-Thr, Gln-Thr, Glu-Thr, Ile-Thr, Pro-Thr, and Trp-Thr in TMV coat protein; two each of Asp-Thr, Leu-Thr, and Thr-Thr, and one each of Ala-Thr, Asn-Thr, Gln-Thr, His-Thr, Ile-Thr, Met-Thr, Pro-Thr, and Val-Thr in TM-1 protein. These peptide bonds covered all possible combinations except Lys-Thr.

Figure 5 shows the cleavage yields of two synthetic dipeptides, Gly-Thr and Gly-Ser, with vapors from various concentrations of aqueous $C_3F_7CO_2H$ at 50°C for 24 h. The

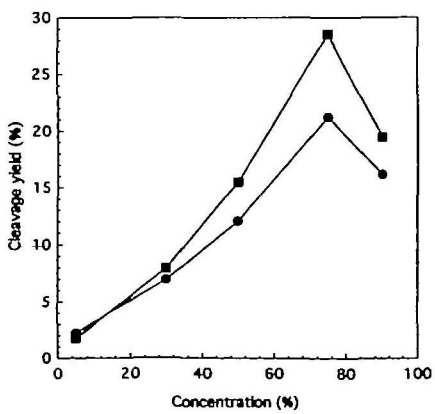

Fig. 5. Gly-Thr cleavage reaction with aqueous $C_3F_7CO_2H$ vapor. Two dipeptides, Gly-Thr (■) and Gly-Ser (●), were exposed to vapors from $C_3F_7CO_2H$ aqueous solutions of various concentrations containing 5% DTT at 50°C for 24 h. The cleavage yields were determined by amino acid analysis of the products.

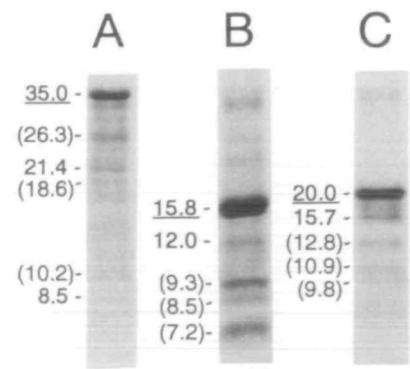

Fig. 6. SDS/Tricine/PAGE patterns of the Gly-Thr cleavage products. Three proteins were treated with aqueous $C_3F_7CO_2H$ vapors containing 5% DTT for 24 h, as follows: lane A, alcohol dehydrogenase was exposed to a vapor of 90% $C_3F_7CO_2H$ at 30°C; lane B, CGMMV coat protein was exposed to a vapor of 5% $C_3F_7CO_2H$ at 40°C; lane C, TMV coat protein was exposed to a vapor of 75% $C_3F_7CO_2H$ at 30°C. The products were applied to SDS/Tricine/PAGE on 16.5% gel. The yields of the cleaved fragments were estimated to be between 4 and 6% for these three proteins from the optical densities of the stained bands and from the sequence yields. Numbers indicate apparent molecular masses (kDa) of Gly-Thr cleavage fragments, and the numbers with underlines indicate the intact proteins. Numbers in parentheses indicate the observed bands for Ser-N cleavage conditions as shown in Fig. 4 (lanes A, B, and C).

cleavage yields of Gly-Thr were higher than those of Gly-Ser for all acid concentrations between 30 and 90%, and the maximal yields for both peptides were observed at 75%. Moreover, 75% acid vapor resulted in the largest difference between the cleavage yields of Gly-Thr and Gly-Ser. These data clearly indicated that the Gly-Thr peptide bond is, unexpectedly, more acid-labile than Gly-Ser bond, which is known to be one of the most acid-labile peptide bonds.

The same three proteins, which contain one or more Gly-Thr peptide bonds, were exposed to acid vapor under the various conditions: 90% $C_3F_7CO_2H$ at 30°C for 24 h for alcohol dehydrogenase; 5% $C_3F_7CO_2H$ at 40°C for 24 h for CGMMV coat protein; 75% $C_3F_7CO_2H$ at 30°C for 24 h for TMV coat protein. The reaction products were separated by SDS/Tricine/PAGE and the peptide fragments were sequenced. The bands whose molecular masses are marked in Fig. 6 had essentially the same sequences as those listed in Table I. All expected fragments produced by cleavage of Gly-Thr bonds were observed for these three proteins: 21.4 kDa (142-Gly-Thr-143) and 8.5 kDa (263-Gly-Thr-264) for alcohol dehydrogenase; 12.0 kDa (32-Gly-Thr-33) for CGMMV coat protein; 15.7 kDa (135-Gly-Thr-136) for TMV coat protein.

Based on the N→O shift mechanism, N-side peptide bonds of serine residues are supposed to be more labile than those of threonine. The above acid-lability of Gly-Thr,

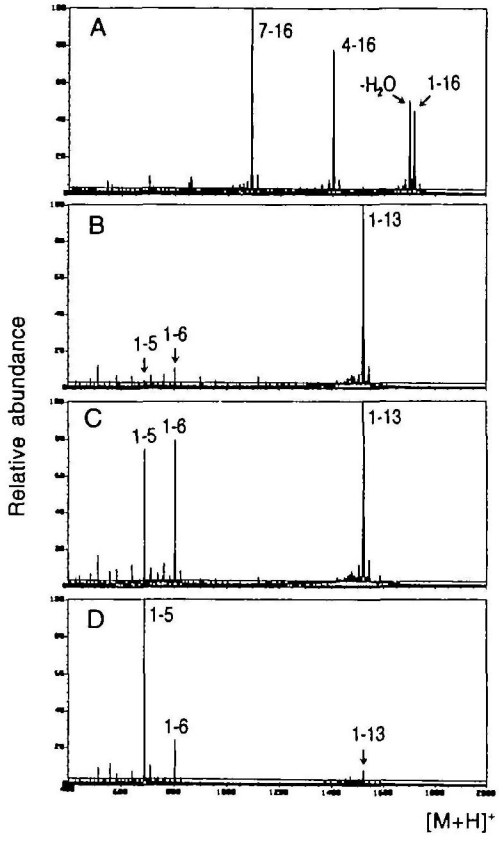

Fig. 7. **FAB mass spectra of Asp-C cleavage products.** (A) The hexadecapeptide, Glu-Ala-Asp-Lys-Ala-Asp-Val-Asn-Val-Leu-Thr-Lys-Ala-Lys-Ser-Gln, was exposed to the vapor of 0.2% $C_3F_7CO_2H$ aqueous solution containing 5% DTT at 90°C for 4 h. A tridecapeptide, Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly, was exposed to the vapor at 90°C for (B) 4 h, (C) 8 h, and (D) 24 h. The cleavage yield for Asp-C estimated from the peak height was 65% in Fig. 7A. In B, C, and D, the yields for Asp-C were 9, 31, and 18% for peaks 1-6 and those for Asp-N were 2, 29, and 77% for peaks 1-5, respectively.

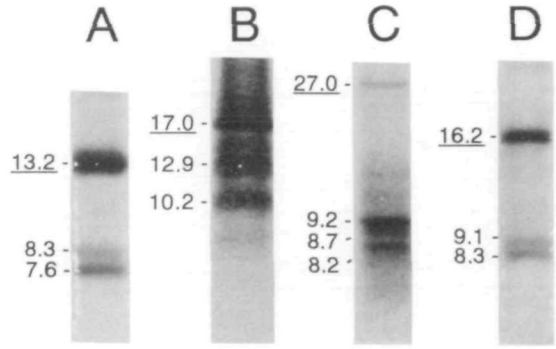

Fig. 8. **SDS/Tricine/PAGE patterns of the Asp-C cleavage products.** Horse heart cytochrome *c*, cormorant myoglobin and snake venom TM-1 protein were exposed to the vapor of 0.2% $C_3F_7CO_2H$ aqueous solution containing 5% DTT at 90°C for 4 h. Calf thymus histone H2A was exposed to the same acid vapor at 90°C for 8 h. The products were subjected to SDS/Tricine/PAGE with 16.5% gel. Lane A, the cleavage product of cytochrome *c*; lane B, that of myoglobin; lane C, that of TM-1; lane D, that of histone H2A. Numbers indicate apparent molecular masses (kDa) of respective bands, and the bands with underlined numbers indicate the intact proteins. The yields of the fragments were estimated to be 10, 50, 90, and 10% for lanes A, B, C, and D, respectively. In lane B, the yield was also estimated from the sequence data as 50%.

TABLE II. **Sequences of the Asp-C cleavage products of proteins.** Proteins were treated with 0.2% $C_3F_7CO_2H$ vapor containing 5% DTT at 90°C for 4 h (cytochrome *c*, myoglobin, and TM-1) or 8 h (histone H2A). Apparent molecular masses correspond to those in Fig. 8. * stands for the C-terminal residue of each protein.

Protein (kDa)	Apparent molecular mass (kDa)	Preceding residue	N-terminal sequence	Possible C-terminal cleaved site ↓
Cytochrome *c*	8.3	*N*-acetyl	Not detected	-Asp-Ala-51
(13.2)	7.6	50-Asp	Ala-Asn-Lys-Asn-Lys-	*-104
Myoglobin	12.9	35-Asp	His-Pro-Glu-Thr-Leu	*-153
(17.0)	12.9	41-Asp	Arg-Phe-Glu-Arg-Phe	*-153
	10.2	60-Asp	Leu-Lys-Lys-His-Gly	*-153
TM-1	9.2	120-Asp	Pro-Lys-Phe-Ser-Val-	*-203
(27.0)	8.7	120-Asp	Pro-Lys-Phe-Ser-Val-	-Asp-Asn-195
	8.2	44-Asp	Met-Tyr-Lys-Pro-Leu-	-Asp-Pro-121
Histone H2A	9.1	*N*-acetyl	Not detected	-Asp-Asn-93
(16.2)	8.3	72-Asp	Asn-Lys-Lys-Thr-Arg-	*-129

therefore, may suggest a specific cleavage. Further experiments are in progress to study this odd observation.

Aspartic Acid C-Side Cleavage—Preliminary experiments indicated that use of vapors from low concentrations of aqueous $C_3F_7CO_2H$ at high temperature such as 90°C was favorable for the specific cleavage at the C-side of aspartic acid. Acid vapor concentrations higher than 1.0% introduced other acid-labile peptide bond cleavages. The acid concentration of 0.2% was chosen for this specific Asp-C cleavage; and while 0.1% may be used, the reaction was observed to be irreproducible by the present double-tube method.

The hexadecapeptide Glu-Ala-Asp-Lys-Ala-Asp-Val-Asn-Val-Leu-Thr-Lys-Ala-Lys-Ser-Gln was again used for this test. With the vapor of 0.2% $C_3F_7CO_2H$, cleavages at the C-sides of Asp-3 and Asp-6 were clearly observed but cleavages of the N-side of Ser-15 and other acid-labile peptide bonds were not observed (Fig. 7A).

In Fig. 7, B to D, a tridecapeptide, Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly, was exposed to 0.2% acid vapor at 90°C for 4, 8, or 24 h. Aspartic acid C-side cleavage was first observed (Fig. 7B), followed by the N-side cleavage (Fig. 7, C and D), showing that the reaction

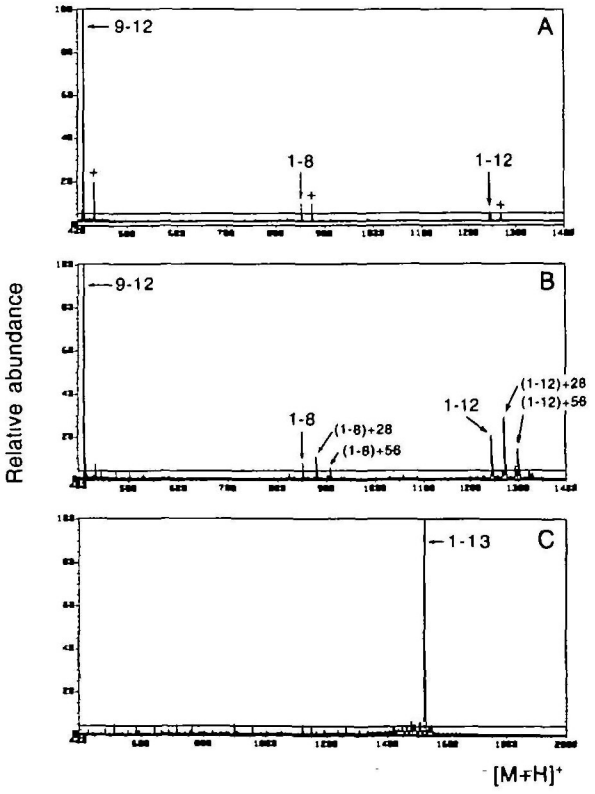

Fig. 9. **FAB mass spectra of the Asp-Pro cleavage products.** (A) A dodecapeptide, Val-Ser-Ser-Asn-Ile-Ser-Glu-Asp-Pro-Val-Pro-Val, was exposed to the vapor of 0.2% $C_3F_7CO_2H$ aqueous solution containing 5% DTT at 60°C for 24 h. + indicates the sodium adduct ion (+22) to the respective peaks. The cleavage yield was estimated to be 96%. (B) The same dodecapeptide was incubated in 70% (v/v) formic acid aqueous solution at 25°C for 72 h. The cleavage yield was 66%. (C) The tridecapeptide, Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly, was exposed to the vapor under the same conditions as in experiment (A), and no fragmentation was observed.

velocities of Asp-C and Asp-N cleavages differ and that the first Asp-C cleavage activates the Asp-N cleavage reaction.

The vapor of 0.2% $C_3F_7CO_2H$ aqueous solution was chosen as the acid concentration for the Asp-C cleavage at the temperature 90°C for 24 h. Temperatures between 80 and 100°C may be used and the reaction time may be adjusted between 4 and 24 h.

Four proteins, cytochrome *c*, cormorant myoglobin, snake venom TM-1, and histone H2A, were exposed to the vapor of aqueous 0.2% $C_3F_7CO_2H$ for 4, 4, 4, and 8 h, respectively, and the electrophoresis patterns of these products are shown in Fig. 8. The bands were sequenced and the results are summarized in Table II.

The Asp-C cleavage of cytochrome *c* resulted in the appearance of 7.6 and 8.3 kDa bands (Fig. 8A). The N-terminal sequence of the 7.6 kDa band indicates the cleavage at the 50-Asp-Ala-51 peptide bond to produce a fragment from the 51st residue to the C-terminus. The residual N-terminal fragment (1-50, 8.3 kDa) also migrated to a similar position but could not be sequenced because of the blocked N-terminus. This protein contains only three aspartic acids, of which only Asp-Ala (50-51) was cleaved, while cleavages of the other two aspartyl bonds, 2-Asp-Val-3 and 93-Asp-Leu-94 were not observed. Five asparagine residues in this protein were not cleaved under the present conditions. This protein also contains the other acid-labile amino acids, 12 glycine residues including the unstable peptide bond Gly-Gly. Cleavages at these peptide bonds were not observed.

The Asp-C cleavage of myoglobin resulted in five bands (Fig. 8B). The N-terminus of the 10.2 kDa band indicated a

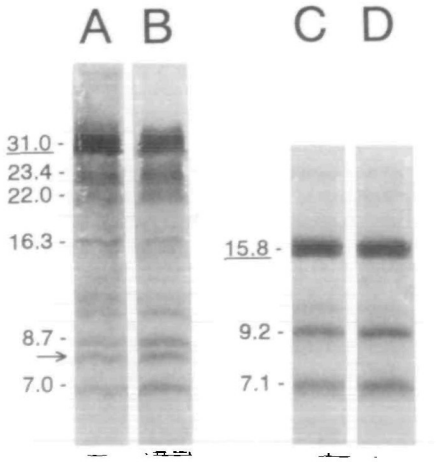

Fig. 10. **SDS/Tricine/PAGE patterns of the Asp-Pro cleavage products.** Bovine carbonic anhydrase II and CGMMV coat protein were cleaved with the vapor from 0.2% $C_3F_7CO_2H$ aqueous solution containing 5% DTT at 60°C for 16 h. These proteins were also incubated in 70% formic acid aqueous solution at 25°C for 72 h. The products were applied to SDS/Tricine/PAGE with 16.5% gel. Lane A, the cleavage product of carbonic anhydrase with 0.2% $C_3F_7CO_2H$ vapor; lane B, the cleavage product of the same protein with 70% formic acid solution; lane C, the cleavage product of CGMMV coat protein with 0.2% $C_3F_7CO_2H$ vapor; lane D, the cleavage product of the same protein with 70% formic acid solution. Numbers indicate apparent molecular masses (kDa) of respective bands, and the bands with underlined numbers indicate the intact proteins. A band marked with → was observed for the protein preparation without acid treatment (data not shown). The yields of cleaved fragments were about 10%.

TABLE III. **Sequences of the Asp-Pro cleavage products of proteins.** Proteins were treated with 0.2% $C_3F_7CO_2H$ vapor containing 5% DTT at 60°C for 16 h. Apparent molecular masses correspond to those in Fig. 10. * stands for the C-terminal residue of each protein.

Protein (kDa)	Apparent molecular mass (kDa)	Preceding residue	N-terminal sequence	Possible C-terminal cleaved site ↓
Carbonic anhydrase II	23.4	40-Asp	Pro-Ala-Leu-Lys-Pro-	*-259
(31.0)	22.0	*N*-acetyl	Not detected	-Asp-Pro-179
	16.3	40-Asp	Pro-Ala-Leu-Lys-Pro-	-Asp-Pro-179
	8.7	178-Asp	Pro-Gly-Ser-Leu-Leu-	*-259
	7.0	*N*-acetyl	Not detected	-Asp-Pro-41
CGMMV coat protein	9.2	*N*-acetyl	Not detected	-Asp-Pro-99
(15.8)	7.1	98-Asp	Pro-Ser-Asn-Pro-Thr-	*-160

cleavage at 60-Asp-Leu-61 peptide bond. This band was predicted to be a fragment from the 61st residue to the C-terminus of the original protein. The 12.9 kDa band was found to have two N-termini, which indicated two cleavages, at the 35-Asp-His-36 and 41-Asp-Arg-42 bonds. Both fragments were predicted to contain the C-terminus of the original protein. The remaining three bands should be also attributable to fragments resulting from the Asp-C cleavages (Miyazaki, K. and Tsugita, A., unpublished).

The Asp-C cleavage of TM-1 resulted in three bands, 9.2, 8.7, and 8.2 kDa (Fig. 8C). The sequence of the 9.2 kDa band showed the cleavage at 120-Asp-Pro-121, and the C-terminus of this fragment was predicted to be that of the original protein. The 8.7 kDa band had the same cleavage site at the N-terminus, and the C-terminus was possibly produced by the cleavage at 194-Asp-Asn-195. The other band, 8.2 kDa, was produced by cleavage of the peptide bond 44-Asp-Met-45 as the N-terminus and probably by cleavage of the peptide bond 120-Asp-Pro-121 as its C-terminus. This protein contains 17 aspartic acid residues including one Asp-Pro bond.

In the case of histone H2A, essentially two bands were observed around 9 kDa (Fig. 8D). The lower band had the N-terminal sequence starting at the 73rd Asn residue of this protein, indicating the peptide 73-129, and the upper band showed the blocked N-terminus, indicating the N-acetyl peptide 1-72. This protein has two aspartic acids, Asp-72 and Asp-90, six asparagines, 14 glycines and four serine residues, and the Asp-C cleavage was observed at 72-Asp-Asn-73 but not at 90-Asp-Glu-91.

In the case of protein cleavage, the reaction conditions may be tested with small amounts of proteins by SDS/PAGE and silver staining by changing the reaction time while maintaining both acid concentration and temperature.

As shown by the above experiments, the Asp-Pro bond is cleaved by the conditions used for Asp-C cleavage.

Aspartyl-Proline Bond Cleavage—The Asp-Pro bond in peptides and proteins is known to be the most acid-labile one (*5*), and this property has been widely used for specific peptide fragmentation.

The peptides Val-Ser-Ser-Asn-Ile-Ser-Glu-Asp-Pro-Val-Pro-Val, which has an Asp-Pro peptide bond, and Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly, which has no Asp-Pro bond but an Asp-Ala bond, were exposed to the vapor of 0.2% $C_3F_7CO_2H$ for 16 h at 60°C. These conditions were milder than those used for the general Asp-C cleavage.

In the former peptide, only the Asp-Pro bond was cleaved, resulting in two peptide fragments, 1-8 and 9-12, and no side reaction was observed (Fig. 9A). The former peptide was also incubated in 70% aqueous (v/v) formic acid at 25°C for 72 h (Fig. 9B). These conventional liquid-phase conditions for Asp-Pro cleavage have often been used because of the high protein solubility. Two molecular ions corresponding to the intact peptide and the cleaved product, 1-8, appeared to be accompanied with their additional 28 and 56 ($=28\times2$) ions. This observation indicates that one and two formyl groups attached to the peptides during the reaction.

In the latter peptide, a tridecapeptide, no cleavage of peptide bond Asp-Ala was observed under the present conditions (Fig. 9C), but this bond was cleaved by the exposure to the acid vapor under the conditions used in Fig. 7. The formic acid solution also did not cleave the tridecapeptide (data not shown).

Two proteins, carbonic anhydrase II and CGMMV coat protein, were exposed to the vapor of 0.2% aqueous $C_3F_7CO_2H$ containing 5% DTT at 60°C for 16 h, and were also incubated in 70% formic acid at 25°C for a longer time, 72 h. Both products were analyzed by SDS/Tricine/PAGE (Fig. 10). Similar band patterns were obtained by the two different acid treatments for both proteins. The results of N-terminal sequence analysis of the peptide fragments are summarized in Table III.

Carbonic anhydrase II has a blocked N-terminus and contains two Asp-Pro bonds, 40-41 and 178-179. The SDS/Tricine/PAGE of the acid-cleavage mixtures, however, gave more than ten faint bands by both acid treatments, which may be due to nonspecific peptide bond cleavages. Five major fragments from acid vapor cleavage were sequenced from the N-termini. The 23.4 kDa band had the N-terminal sequence from the 41st proline residue of the intact protein. This result and the apparent molecular mass of the band indicate that the peptide 41-259 was produced by the cleavage. The 22.0 kDa band had a blocked N-terminus, which indicates that the band is the N-terminal fragment of the protein. The C-terminus of the band may be produced by cleavage at 178-Asp-Pro-179. The 16.3 and 8.7 kDa bands were shown to be the peptides 41-178 and 179-259, respectively, from the sequences and the apparent molecular masses. The 7.0 kDa band was also predicted to be an N-terminal fragment, 1-40, from the blocked N-terminus and the molecular mass. This protein has 16 aspartic acid residues. Among them, two Asp-Pro, one Asp-Asp, two Asp-Ser, two Asp-Gly, four Gly-Ser and two Gly-Thr are acid-labile peptide bonds, but only the Asp-Pro bonds were observed to be substantially cleaved.

The CGMMV coat protein has one Asp-Pro bond (98-99), and its cleavage resulted in two fragments, 9.2 and 7.1 kDa, on the SDS-PAGE. The N-terminal sequence and molecular mass of the lower band indicate the peptide fragment

99-160, and the upper band had the blocked N-terminus indicating the N-acetyl peptide fragment 1-98. This protein contains 10 aspartyl bonds including one each of the acid-labile Asp-Pro, Asp-Asp, Asp-Asn, Asp-Ser, and Gly-Thr bonds. Among them, only Asp-Pro was observed to be cleaved.

The Asp-Pro cleavage reaction using 0.2% $C_3F_7CO_2H$ vapor caused no side reaction such as the acylation observed in the conventional formic acid method, shown in Fig. 9B. Moreover, the vapor reaction time is much shorter. These advantages may be favorable to micro and rapid sequencing of protein. Comparison of the yields of these two Asp-Pro cleavage methods revealed that the $C_3F_7CO_2H$ vapor method gave somewhat higher yields for peptide bond cleavages in hydrophobic regions, while the conventional formic acid solution resulted in higher cleavage yields for peptide bonds in hydrophilic regions. This difference may be due to differences in the reagents, the phase, or the hydrophobicity of the acids. In addition, the vapor reaction resulted in less contamination than that in the liquid phase. Furthermore, application of this vapor method to an acid-insoluble protein, *m*-hydroxybenzoate-4-hydroxylase from *Comamonas testosteroni*, which is insoluble even in 70% formic acid, resulted in more efficient Asp-Pro bond cleavage than the liquid method (*14*).

Conclusions—We propose the following conditions for specific cleavages of peptides and proteins. (1) A vapor of 75% aqueous $C_3F_7CO_2H$ cleaves peptide bonds on the N-sides of serine residues when applied to peptides at 50°C for 24 h or to proteins at 30°C for 24 h. Cleavages of the Gly-Thr bond were also observed in addition to Asp-Pro bond cleavage. Cleavages of Pro-Ser and Ser-Ser were not observed. (2) A vapor of 5-90% $C_3F_7CO_2H$ cleaves Gly-Thr peptide bonds when applied to proteins at 30 to 40°C for 24 h. (3) A vapor of 0.2% $C_3F_7CO_2H$ cleaves the aspartyl bond when applied at 90°C for 4 h in peptides or 4-8 h in protein. (4) At 60°C for 16 h, a vapor of 0.2% $C_3F_7CO_2H$ cleaves the Asp-Pro bond of peptides and proteins. Other aspartyl peptide bonds are stable under these conditions.

These methods complement enzymatic fragmentation, and in some cases they may be the preferred methods. The volatile reagent may be easily removed, making the sample available for subsequent analysis and avoiding contamination of the sample as much as possible.

In chemical cleavage reactions, the pursuit of cleavage selectivity normally entails use of milder conditions that may sacrifice cleavage yields, while the pursuit of a high cleavage yield may cause loss of cleavage selectivity. One interesting aspect of these reactions is their application to proteins, and in this type of work one should test conditions on a small scale and, in general, choose cleavage selectivity over high cleavage yield.

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REFERENCES

1. Tsugita, A., Takamoto, K., Kamo, M., and Iwadate, H. (1992) C-Terminal sequencing of protein. A novel partial acid hydrolysis and analysis by mass spectrometry. *Eur. J. Biochem.* **206**, 691-696
2. Sanger, F. and Thompson, E.O.P. (1953) The amino-acid sequence in the glycyl chain of insulin. 1. The identification of lower peptides from partial hydrolysates. *Biochem. J.* **53**, 353-366
3. Partridge, S.M. and Davis, H.F. (1950) Preferential release of aspartic acid during the hydrolysis of proteins. *Nature* **165**, 62-63
4. Inglis, A.S. (1983) Cleavage at aspartic acid. *Methods Enzymol.* **91**, 324-332
5. Poulsen, K., Fraser, K.J., and Haber, E. (1972) An active derivative of rabbit antibody light chain composed of the constant and the variable domains held together only by a native disulfide bond. *Proc. Natl. Acad. Sci. USA* **69**, 2495-2499
6. Landon, M. (1977) Cleavage at aspartyl-prolyl bonds. *Methods Enzymol.* **47**, 145-149
7. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
8. Tsugita, A., Ataka, T., and Uchida, T. (1987) Approaches for submicrosequencing. *J. Protein Chem.* **6**, 121-130
9. Schägger, H. and von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**, 368-379
10. Ploug, M., Jensen, A.L., and Barkholt, V. (1989) Determination of amino acid compositions and NH_2-terminal sequences of peptides electroblotted onto PVDF membranes from Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis: application to peptide mapping of human complement component C3. *Anal. Biochem.* **181**, 33-39
11. Hewick, R.M., Hunkapiller, M.W., Hood, L.E., and Dreyer, W.J. (1981) A gas-liquid solid phase peptide and protein sequenator. *J. Biol. Chem.* **256**, 7990-7997
12. Kamo, M., Takamoto, K., Kawakami, T., and Tsugita, A. (1995) Selective cleavage of protein with a vapor from perfluoric acid aqueous solution. *Protein Sci.* **4**, Suppl. 2, 158
13. Tsugita, A., Kamo, M., Kawakami, T., and Ohki, Y. (1996) Two-dimensional electrophoresis of plant proteins and standardization of gel patterns. *Electrophoresis* **17**, 855-865
14. Scott, R.P., Jr., Tsugita, A., and Hosokawa, K. (1997) A novel m-hydroxybenzoate-4-hydroxylase from *Comamonas testosteroni*. *Res. Commun. Biochem. Cell. Mol. Biol.*, in press