J. Biochem. 120, 29-34 (1996)

Systematic Peptide Fragmentation of Polyvinylidene Difluoride(PVDF)-Immobilized Proteins Prior to Microsequencing

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Received for publication, September 20, 1995

The sequential *in situ* digestion of proteins immobilized on polyvinylidene difluoride (PVDF) has been systematically designed and optimized. The method consists of immobilization of the proteins on PVDF, *S*-carboxymethylation, and then successive *in situ* digestions using specific proteases. In order to obtain high yields of the peptide fragments, from which specific amino acid residues connected to the N- or C-terminal of the resulting digestion fragments can be deduced, the cleavages are performed in the following order: (1) *Achromobacter* protease I (API), (2) endoproteinase Asp-N, and (3) trypsin. Procedures for recovering the numerous fragments remaining on the PVDF membrane after the third digestion with trypsin are also discussed. Application of sequential *in situ* digestion for the acquisition of fragments suitable for sequencing from digests of large-molecular-weight proteins is also presented.

Key words: deducible amino acid residues, PVDF membrane, recoveries of digests from PVDF, *S*-alkylation of protein, sequential *in situ* digestion.

Protease digestion of target proteins followed by internal amino acid sequencing of purified peptides has the potential to yield significantly more sequence data than direct N-terminal analysis of intact proteins. This is because N-terminal sequencing gives only a single sequence or in some cases no structural information, due to blockage at the α-amino group. Prior to protease digestion, proteins must usually be reduced and *S*-alkylated in order to obtain efficient fragmentation. However, if only a limited amount of a protein is available (less than 100 pmol), the protein can be directly digested without *S*-alkylation. This procedure avoids sample loss during the reactions and subsequent purifications, but it has been shown that non-alkylated proteins purified by gel electrophoresis give complicated peptide maps after protease digestion and recoveries of the generated fragments can be poor. We have previously shown that these problems can be overcome by the quantitative reduction and *S*-carboxymethylation of the protein immobilized on PVDF (*1*). However, occasionally only a few peptide fragments are obtained from PVDF membranes, even after *in situ* *S*-carboxymethylation and protease digestion. This often occurs because many of the fragments generated by protease digestion are too hydrophobic to be extracted from the PVDF membrane. In this paper we describe a method for obtaining more peptide fragments by sequential protease digestions of fragments on PVDF membranes. In addition, we demonstrate that the systematic stepwise digestion of PVDF-immobilized proteins often gives superior results to simple digestion.

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Abbreviations: AU, absorption units; AUFS, absorption units full scale; PVDF, polyvinylidene difluoride; rh-G-CSF, recombinant human granulocyte colony-stimulating factor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

MATERIALS AND METHODS

Materials—*Achromobacter* protease I, iodoacetic acid, acetonitrile, and 2-propanol (HPLC grade) were purchased from Wako Pure Chemical; Polyvinylpyrrolidone (PVP-40) and Ponceau S from Sigma; endoproteinase Asp-N (sequence grade) from Boehringer Mannheim; trypsin (TPCK-treated) from Worthington Biochemical; arginyl-endopeptidase and asparaginyl-endopeptidase from Takara; PVDF membrane filters (Immobilon-P^{SQ}) from Millipore; and (Pro Blott) from Applied Biosystems. Recombinant human granulocyte colony-stimulating factor (rhG-CSF) was provided by the Kirin Pharmaceutical Laboratory. All other chemicals were of the highest grade commercially available.

Immobilization of Proteins on PVDF Membranes—Proteins in solution were bound to PVDF membranes by spotting onto the membrane. Proteins separated by gel electrophoresis were electroblotted onto PVDF membranes using Tris-ε-aminocaproic acid-methanol buffer (*2*) and stained with Ponceau S (*3*).

Reduction and S-Carboxymethylation of PVDF-Immobilized Proteins—PVDF-immobilized proteins were reduced at 25°C for 1 h in 200 μl of 8 M guanidine containing 0.5 M Tris-HCl (pH 8.6), 0.3% EDTA (disodium salt), 5% acetonitrile, and 1 mg of DTT. Iodoacetic acid (2.6 mg) was then dissolved in 10 μl of 1 N NaOH, added to the reducing solution and stirred for 15-20 min in the dark. The PVDF membrane was then thoroughly washed with 2% acetonitrile (100 ml × 2) and treated in 0.1% SDS (100 ml) for 5 min with stirring.

In Situ Digestion of PVDF-Immobilized Proteins—Prior to sequential digestions, the protein immobilized on PVDF membrane was treated with PVP-40 (*3*), then the membrane was thoroughly washed with 10% acetonitrile (100 ml) for 15 min using a magnetic stirrer. Before the subdigestions (second or further digestion), the membrane was washed with subdigestion buffer solution. These cleavages were performed without additional PVP-40 treatment. After thorough examinations into the effect of temperature, buffer solution, time and enzyme-to-substrate ratio (E/S), the optimum digestion conditions were determined to be as follows; API (lysylendopeptidase) digestion was performed in 20 mM Tris-HCl buffer (pH 9.0) containing 10% acetonitrile at 37°C for 16 h using an E/S of 1/100 (mol/mol). Digestion with endoproteinase Asp-N was done in 20 mM ammonium bicarbonate (pH 7.8) with 25 mM calcium chloride containing 10% acetonitrile at 40°C for 16 h with an E/S of 1/100 (mol/mol). Tryptic or chymotryptic digestion was performed in 20 mM ammonium bicarbonate (pH 7.8) with 10 mM calcium chloride containing 10% acetonitrile at 25°C for 16 h with an E/S of 1/100 (mol/mol). One picomole of enzyme was used for each cleavage reaction, if less than 100 pmol of sample protein was used.

Peptide Mapping by Reverse-Phase HPLC—Peptides generated by *in situ* endoprotease digestion were subjected to reverse-phase HPLC using a μ-Bondasphere (5μC8-300Å, 2.1×150 mm, Waters) column or a Wakosil-II AR (C18 300Å, 2.0×150 mm, Wako Pure Chemical) column. A Hitachi L6200/6000 liquid chromatograph was used with a Rheodyne (model 7125) sample injector having a 250 μl loop. Peptide elution was done with a linear gradient of solvent B in 30 or 40 min at a flow rate of 0.25 ml/min using the following solutions: solvent A; 0.05% trifluoroacetic acid in water, and solvent B; 0.02% trifluoroacetic acid in 2-propanol: acetonitrile, 7 : 3 (v/v). The fractionated peptides were collected manually by monitoring the absorbance at 205 or 215 nm with or without baseline normalization using a Uni-2 (Union).

Amino Acid Sequencing and Composition Analysis—Amino acid sequencing was performed with a Shimadzu PSQ-1. Amino acid analyses were carried out as described (*4*) in order to quantify peptide fragmentation.

RESULTS AND DISCUSSION

An Example of Successive Digestions on PVDF Membrane and Selection of Proteases Suitable for Sequential Digestions—Proteins immobilized on PVDF membranes can be digested with some proteases to the same extent as proteins in solution. However, when *in situ* digestions are performed on PVDF-immobilized proteins, some of the more hydrophobic peptide fragments are not recovered from the membrane. This is normally considered to be a disadvantage of *in situ* digestion when compared to conventional digestion in solution. A distinctive example of this was reported with a 38 kDa protein from porcine epididymal sperm which possesses *zona pellucida*-binding activity (*5*). This protein was subjected to SDS-PAGE and subsequent electroblotting. Visualization of the protein with Ponceau S on the blot indicated that more than 50 pmol of protein was present. After digestion with endoproteinase Asp-N, HPLC analysis did not detect any peptide fragments in the buffer solution (Fig. 1a). Based on these results, *in situ* digestion would not be considered to be a generally applicable procedure. It is possible that although some digestion did occur, the resulting peptides remained bound to the PVDF membrane due to their hydrophobicity. If such fragments were retained on the PVDF membrane, recovery may be feasible by reducing their hydrophobicity by "re-digestions" with proteases possessing different substrate specificity from those previously used. This was shown to be the case when the Asp-N fragments of the 38 kDa protein on the transblot membrane were digested again using API. Subsequent HPLC analysis demonstrated that several peptides could then be eluted from the PVDF (Fig. 1b). Therefore, if numerous suitable proteases exist for successive digestions on PVDF membrane, it should be possible to design a multi-step sequential *in situ* digestion protocol to obtain as much useful sequence information as possible.

In order to determine what kinds of proteases are suitable for successive *in situ* digestion, API (lysylendopeptidase), *S. aureus* V8 protease, endoproteinase Asp-N, arginylendopeptidase, asparaginyl-endopeptidase, and trypsin were examined. API, endoproteinase Asp-N, and trypsin efficiently hydrolyzed PVDF-immobilized proteins. Arginylendopeptidase and asparaginylendopeptidase were rather ineffective. V8 protease was required in a relatively high amount (E/S=1/20 to 1/5, mol/mol) for sufficient digestion. In addition, if API or endoproteinase Asp-N had been used previously, the V8 protease itself was partially digested by these enzymes, resulting in contaminating V8 peptide fragments. Thus, API, endoproteinase Asp-N, and trypsin were concluded to be the most suitable proteases for studies involving sequential *in situ* digestion.

Order of Protease Digestion for Systematic Fragmentation—In order to obtain as much informative sequence data as possible, the order in which proteases were to be used was chosen according to the following criteria. (1) Limited number of cleavage sites in each digestion to minimize the number of fragments generated, preventing digests from

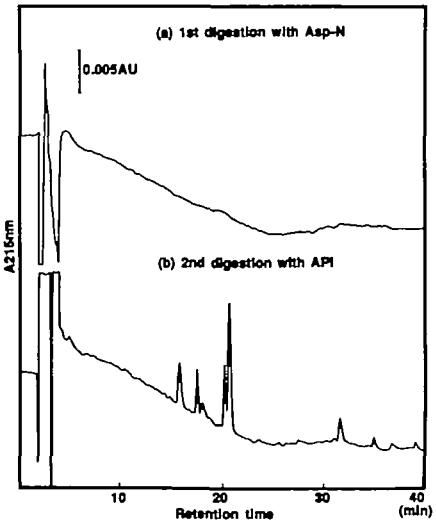

Fig. 1. **Peptide maps from reverse-phase HPLC of a porcine epididymal sperm protein (38 kDa) having *zona pellucida*-binding activity.** Approximately 80 pmol of this was first digested with 1 pmol of endoproteinase Asp-N (a) and then cleaved with 1 pmol of API (b).

co-eluting on HPLC as much as possible. (2) Deduction of the amino acid residues connected to the termini of analyzed peptides as far as possible. The significance of the later point arises from the fact that microsequencing in reality usually gives sequences in which amino acids are determined ambiguously or undetermined ones are included, so in some cases, using deducible amino acids connected to analyzed peptides can be practically advantageous for success in subsequent DNA cloning. Taking only the first point into consideration, either API or Asp-N is suitable for primary and subsequent second digestions. However, taking into account the second criterion as well, API digestion was determined to be the most suitable for the first digestion, because lysine residues are expected to be connected to the analyzed fragments after API digestion. In addition, lysine residues can also be expected even after the second digestion with Asp-N, if the N-terminal residues of analyzed fragments are not aspartic acid. In contrast, if Asp-N is used for the first digestion, aspartic acid residues are deducible as the residues aligned to the C-termini of the sequenced peptides. However, using N-terminal sequencing alone, it is difficult to define C-terminal residues conclusively. Therefore, the API and Asp-N digestions were done in that order, and trypsin was used for the third digestion. Trypsin normally hydrolyzes both lysyl and arginyl bonds but, in this case, the enzyme only cleaves at arginine residues because all of the lysyl bonds had already been digested with API. Since sequential digestion with these three enzymes provides a method to digest at the C-terminus of lysine, the N-terminus of aspartic acid and the C-terminus of arginine, in that order, some of the amino acid residues connected to the fragments determined can be theoretically deduced as shown in Fig. 2.

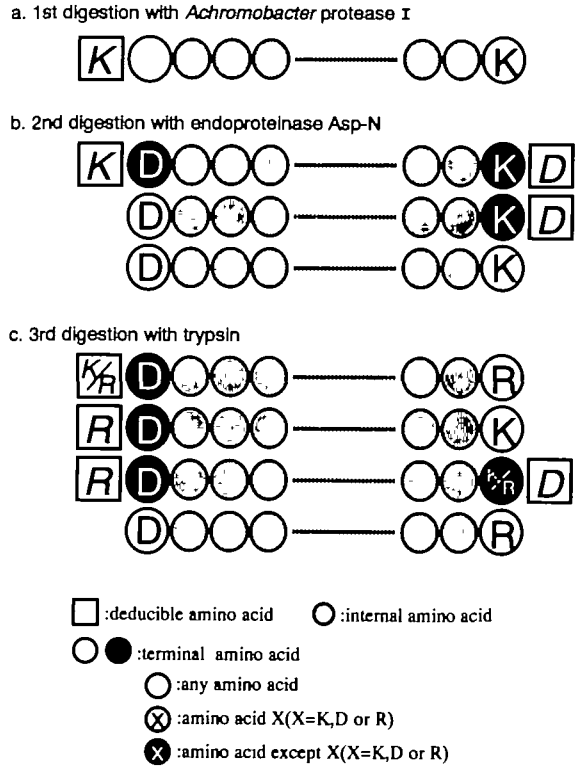

Fig. 2. **Types of internal peptide fragments generated after each digestion and deducible amino acid residues connected to the respective fragments.** (a) From the first digestion with API, lysine is determined to be connected to the N-terminal. (b) From the second digestion with Asp-N, lysine to the N-terminal side and aspartic acid to the C-terminal side are deducible if the N-terminal residue of the fragment is not aspartic acid. In addition, aspartic acid can be connected to the C-terminal, if the N-terminal amino acid is aspartic acid and the C-terminal is not lysine. (c) Some deduction is possible from trypsin digestion for peptides whose N-terminal is not aspartic acid. When the C-terminal is arginine, arginine or lysine is connected to the N-terminal. When the C-terminal residue is lysine, arginine is connected to the N-terminal. When the C-terminal is neither lysine nor arginine, arginine is connected to the N-terminal and aspartic acid is connected to the C-terminal. *NOTE:* Deduction of amino acid residues is not applicable for terminal peptide fragments. If an excess amount of protease is used, some non-specific cleavage may occur, for example, cleavage at the amino side of glutamic acid by endoproteinase Asp-N digestion and chymotrypsin-like cleavage with trypsin.

Recoveries of Fragments after Each Digestion—Recombinant human granulocyte colony-stimulating factor (rh-G-CSF), which is relatively hydrophobic, was chosen as the substrate protein to quantify peptide recoveries from successive digestions. This choice was based on the fact that it is generally accepted to be harder to obtain digests of hydrophobic proteins from PVDF membrane. Figure 3 shows the peptide map of 50 pmol of rh-G-CSF that was immobilized on PVDF by spotting. The fragments obtained from each digestion are assigned to the rh-G-CSF sequence as shown in Fig. 4. In the first digestion with API, 4 of the 5 expected fragments (AP-1 to AP-4) were obtained. The

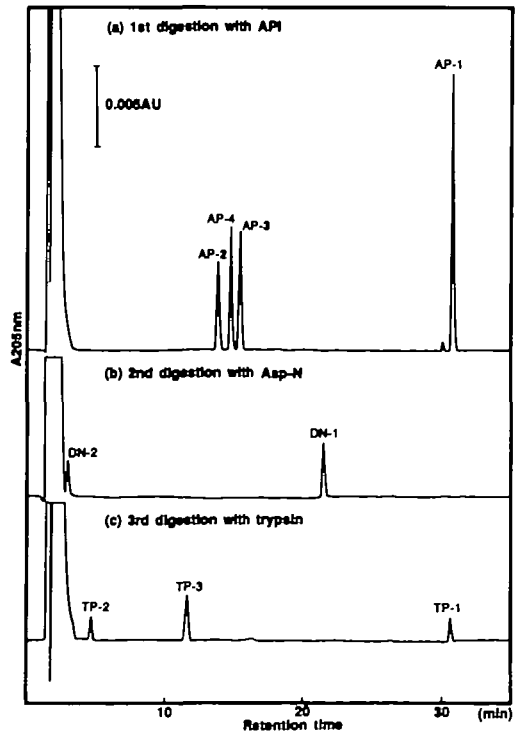

Fig. 3. **Peptide maps from reverse-phase HPLC of rh-G-CSF fragments.** Two hundred picomoles of rh-G-CSF was digested first with 2 pmol of API (a), secondly with 2 pmol of Asp-N (b), and thirdly with 2 pmol of trypsin (c). A quarter of the volume of each digest solution was injected.

largest theoretical peptide ("AP-5") did not appear on the chromatogram and remained bound to the membrane. Two (DN-1 and DN-2) and three peaks (TP-1 to TP-3) were observed on the chromatogram after the second and third digestions with Asp-N and trypsin, respectively. As shown in Table I, relatively large amounts of the fragments AP-2, -3, -4, DN-1, -2, TP-2, and -3 were eluted from the HPLC column using fairly low concentrations of organic solvent (Solvent B). However, the two fragments, AP-1 and TP-1, which were eluted from the HPLC column at relatively high concentrations of Solvent B, were obtained in low yields. The reason for the low recoveries of these two fragments is probably not incomplete digestion, as the peptide bond between Lys17 and Cys18 was comprehensively cleaved to give a high yield of AP-2, for example. Thus, a more likely explanation for the low yields is that the peptides may remain partially bound to the membrane. Alternatively, the fragments may be non-specifically absorbed on various materials, such as the polypropyrene sample tubes. In fact, the recovery of AP-1 was strongly dependent on the sample tubes used, although the recoveries of AP-2 , -3, and -4 were constant. We evaluated recovery yields using several types of commercially available sample tubes, and found that the recovery ranged from 20 to 55%, depending on the tubes. In general, hydrophobic peptides were recovered in lower yields than hydrophilic peptides. This situation also would apply to the same fragments obtained after digestion in solution, and it is one of the distinctive features of sequential *in situ* digestion. Although all digested fragments may be obtained after digestion in solution, any long (hydrophobic) fragments, such as those remaining on the PVDF membrane after *in situ* digestion, must be chromatographed before they can be directly sequenced or subdigested. The extra steps required would result in substantially more sample loss. This problem is not as apparent in the case of *in situ* digestion of the long fragment (AP-5) extending from the 42nd residue to the C-terminus of rh-G-CSF. This peptide remains on the membrane without loss, so that subdigestion can be performed to give digests in high yields even when a small amount of starting material is used.

Another factor affecting the yield of various peptides was the degree of *S*-alkylation of the PVDF-immobilized protein. Previously, we alkylated the blotted samples in 6 M guanidine hydrochloride solution (*1*). However, *S*-carboxymethylation of rh-G-CSF at Cys18 could not be performed quantitatively using this procedure, even if the reaction time or temperature was increased. We found that the recovery of some fragments was reduced due to incomplete alkylation, which was probably caused by insufficient denaturation of the protein. In addition, we found that digestion conditions were significant in order to carry out digestions at specific residues quantitatively with minimum non-specific cleavage. Therefore, we finally determined the optimum conditions for *S*-alkylation and digestions as described in "MATERIALS AND METHODS."

Analysis of the Fragments Remaining on PVDF Membranes—After the third digestion with trypsin, the fragments LCHPEELVLLGHSLGIPWAPLSSCPSQALQLAGCLSQLHSGLFLYQGLLQALEGISPELGPTL and DFATTIWQQMEELGMAPALQPTQGAMPAFASAFQR remained on the PVDF membrane. They could be extracted by using 20% acetonitrile as shown in Fig. 5. The yields of these two fragments, TP-w1 and TP-w2, were approximately 20% of the 200 pmol used as the original starting material, and the yields of these fragments from 50 pmol were much lower (Table I). The phenomenon implies that

TABLE I. **Recoveries of rh-G-CSF fragments after each digestion** ($n=4$).

Digestion	Peptide	Yield from:	
		200 pmol	50 pmol
		(%)	(%)
1st API	AP-1	51.5±3.5	55.4±1.3
	AP-2	76.6±2.8	67.5±3.0
	AP-3	76.5±2.5	72.8±2.9
	AP-4	78.1±2.2	67.8±3.6
	AP-5	0	0
2nd Asp-N	DN-1	81.3±2.6	76.4±4.0
	DN-2	85.8±1.1	47.6±2.6
3rd trypsin	TP-1	5.1±1.5	9.4±1.7
	TP-2	75.4±3.1	40.7±3.5
	TP-3	81.4±2.4	76.4±2.9
	TP-w1	17.4±1.1	3.5±1.5
	TP-w2	21.2±3.7	4.3±3.4

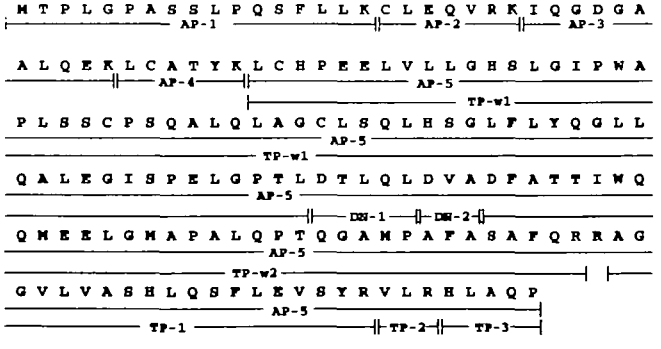

Fig. 4. **Amino acid sequence of rh-G-CSF.** Lines with bars on both ends indicate peptides obtained by sequential *in situ* digestion with *Achromobacter* protease I, Asp-N, and trypsin, and extracted with 20% acetonitrile after the third digestion. The peptide names correspond to the names shown in Figs. 3 and 5. The peptide alignments were identified by amino acid sequencing, compositional analyses and mass spectrometry.

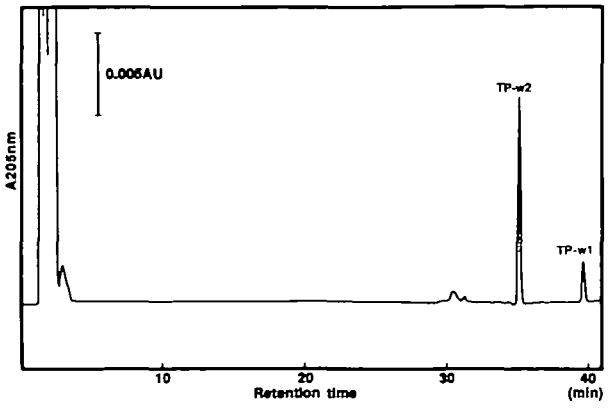

Fig. 5. **Peptide maps from reverse-phase HPLC of rh-G-CSF fragments.** After the third digestion of 200 pmol of rh-G-CSF with trypsin, fragments remaining on the PVDF membrane were extracted with 20% acetonitrile. A quarter of the extract was injected.

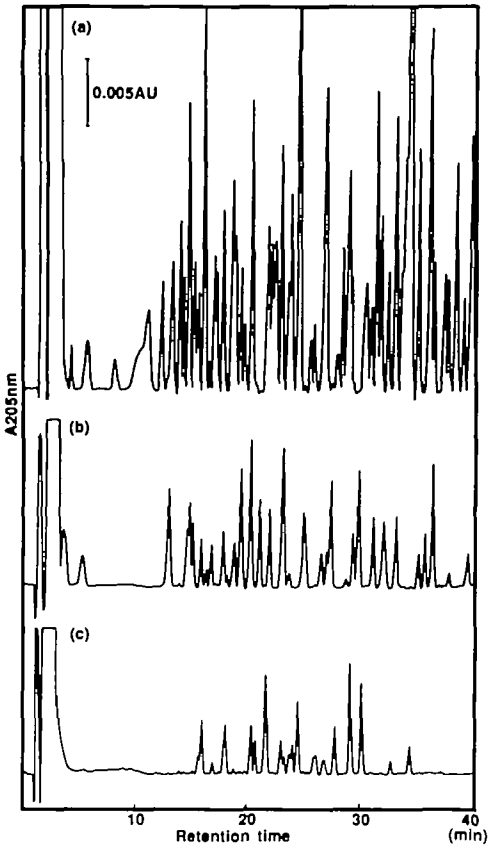

Fig. 6. **Peptide maps from reverse-phase HPLC of a membrane protein (about 400 kDa).** About 50 pmol of a membrane protein was digested first with 1 pmol of API (a), secondly with 1 pmol of Asp-N (b), and thirdly with 1 pmol of trypsin (c). All of each digest solution was injected. Almost all peaks obtained by API digestion contained more than two fragments, whereas more than half of the peaks obtained after Asp-N and trypsin digestions contained a single fragment.

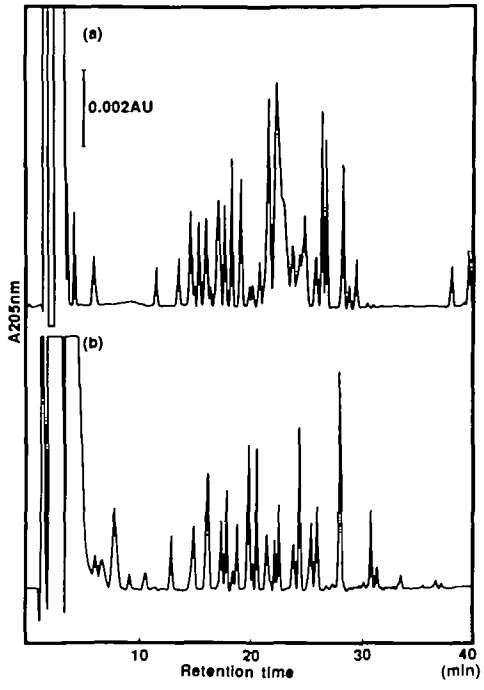

Fig. 7. **Peptide maps from reverse-phase HPLC of a 135 kDa signal transduction protein.** About 15 pmol of the protein was digested first with 1 pmol of API (a) and secondly with 1 pmol of Asp-N (b) about 1 year after the API digestion. All of each digest solution was injected. The yields of both digests were almost the same, as judged from the initial yields of the fragments in Edman degradations on a gas-phase sequencer.

the degree of non-specific absorption of hydrophobic fragments on experimental materials is higher when a lower quantity of starting material is subjected to digestion. As an alternative strategy, we attempted further subdigestions. Since the fragments contained many hydrophobic residues, subdigestion with α-chymotrypsin and thermolysin were examined. Although thermolysin did not react well *in situ*, digestion with α-chymotrypsin yielded peptide fragments (data not shown).

Other Advantages of Sequential In Situ Digestion—We found several advantages to this method while applying it to many different proteins. For example, digestion in solution of a protein with a molecular weight greater than 100 kDa would yield too many fragments to separate by reverse-phase HPLC. In this case, it might be very difficult to obtain much useful sequence information. However, several single fragments could probably be purified after subdigestions by using the method of sequential *in situ* digestion. A sequential digestion of a membrane protein (about 400 kDa) supported this proposal (Fig. 6). Another merit of this procedure is that some peptide fragments suitable for sequencing can be obtained from small amounts of membrane-bound digests even after long-term storage. For example, we had digested about 15 pmol of a novel signal transduction protein (135 kDa) on a PVDF membrane with API (Fig. 7a), and the digest remaining on the membrane was stored in water at 4°C. In Fig. 7b the peptide map resulting from endoproteinase Asp-N digestion of the digest on the membrane that had been stored for about 1 year is shown, although partial degradation must be taken into account in this instance.

Concluding Remarks—To investigate its versatility, the described method of sequential *in situ* digestion was applied to several proteins with unknown sequences, including approximately 70 pmol of mouse glycosylation inhibiting factor (12.5 kDa) (*6*), 50 pmol of bovine holocarboxylase synthetase (63 kDa) (*7*), 20 pmol of rat Ras GTPase-activation protein (100 kDa) (*8*), 10 pmol of HeLa zinc regulatory factor (116 kDa) (*9*), and 10 pmol of rat thrombopoietin (19 kDa) (*10*). We had reasonable success in obtaining digests with all the proteins except thrombopoietin. Only three fragments were obtained from thrombopoietin, one from the first digestion with API and the others from the third digestion with trypsin. These three fragments were located at the N-terminus and close to the C-terminal region. The internal portion remained unextractable in spite of the existence of several Lys, Asp, and Arg residues. This portion was probably not recovered because the proteases cannot digest the internal residues due to the strong hydrophobic binding of this protein to the membrane.

Currently, it is becoming increasingly important to determine internal amino acid sequences for cDNA cloning and to learn the chemical structure of proteins themselves,

including the identification of blocked N-termini, side chain modifications, and the presence of carbohydrate moieties. This has increased the demand for the development of novel and improved microstructural analytical techniques in order to obtain high yields of a maximal number of peptides from a given protein sample. The sequential *in situ* digestion procedure alleviates many of the recovery problems, facilitating the attainment of reliable sequence information from which stringent oligonucleotide probes can be designed, though several difficulties remain. For example, additional work is required to obtain adequate sequence information from the strongly hydrophobic fragments observed during the digestion of thrombopoietin (*10*); this problem is being addressed in our laboratories.

We thank Ettore Appella of NCI, NIH (USA), and Paul D. Fraser of our laboratories for critical readings of the manuscript, and Katsuya Okawa of our laboratories for quantitative amino acid analyses.

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