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Internal amino acid sequence analysis of proteins separated by one- or two-dimensional gel electrophoresis after *in situ* protease digestion on nitrocellulose

(protein sequence analysis/electroblotting/peptide mapping/Edman degradation)

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ABSTRACT **We have developed a general two-step method for obtaining peptide fragments for sequence analysis from picomole quantities of proteins separated by gel electrophoresis. After separation by one- or two-dimensional polyacrylamide gel electrophoresis, proteins are electrophoretically transferred (electroblotted) onto nitrocellulose, the protein-containing regions are detected by reversible staining and are cut out, and each protein is digested *in situ* by proteolytic enzymes such as trypsin or staphylococcal V-8 protease. The resulting peptide fragments are separated by narrow-bore reverse-phase HPLC, collected, and sequenced in a gas-phase sequenator. Excellent peptide recoveries and the absence of extraneous contaminants in the separation of the peptide fragment mixture allow the generation of extensive internal sequence information from picomole amounts of protein. The method thus overcomes the problem of obtaining amino acid sequence data from N-terminally blocked proteins and provides multiple, independent stretches of sequence that can be used to generate oligonucleotide probes for molecular cloning and/or used to search sequence data bases for related proteins. This method has been successfully applied to the routine amino acid sequence analysis of a wide range of proteins isolated from one- and two-dimensional polyacrylamide gels.**

Protein sequence analysis is central to modern biological research. Amino acid sequence data can aid in gene isolation either through the use of synthetic oligonucleotides (1) or through the use of antisera of predetermined specificity (2). Sequence data can also be used to correlate the gene structure with the expressed polypeptide chain, to map active sites and domain boundaries, to define posttranslational modifications, or to establish evolutionary relationships between proteins (3).

Currently, 20 pmol amounts of protein can give limited N-terminal sequence information using optimized isolation methods (4) and a commercially available gas-phase sequenator (5). It is desirable to develop a method of comparable sensitivity for internal amino acid sequence analysis of proteins for the following reasons:

(*i*) Many proteins are not susceptible to the Edman degradation. This is assumed to be due to artefactual or biosynthetic blocking of the α-amino groups of the proteins. The nature of the modified N terminus is not easily determined and only a few of the modifications can be chemically or enzymatically reversed (6). Therefore, the most general way to obtain sequence information from a protein with a blocked α-amino group is to sequence peptide fragments generated by cleavage of the polypeptide chain.

(*ii*) In the molecular cloning of genes through the use of synthetic oligodeoxynucleotides reverse translated from the protein sequence, it is advantageous to screen appropriate cDNA libraries with either a set of multiple probes or a single probe with minimum degeneracy to reduce the incidence of "false positives." Furthermore, the use of an oligodeoxynucleotide probe derived from the N terminus of the protein requires full-length cDNA clones or tedious and repetitive cloning from random-primed libraries (7). Multiple probes can be used to select long cDNA clones in a background of shorter clones.

(*iii*) Amino acid sequences obtained at the low picomole level (10–20 pmol) are usually relatively short (10–25 amino acid residues). Homology searches of data bases for nonidentical, but related, proteins using such short stretches of amino acid sequence may give rise to numerous false matches. Related proteins can more reliably be found by multiple screening of the data base with a set of peptides derived from one protein.

The highest resolving procedures for the analysis of microgram or submicrogram amounts of proteins are one-dimensional (1D) and two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE) (8, 9). Although many proteins can be identified through their behavior in analytical 2D gel electrophoresis, sequence data are available for only a few of these proteins due to the difficulty of isolating sufficient quantities in a form suitable for sequence analysis (10). In the past, proteins have been recovered from the gel matrix by (electro)elution (11) but they are frequently blocked at the N terminus and are typically contaminated with large amounts of $NaDodSO_4$ and salts, agents that hinder enzymatic cleavage and separation of the resulting peptide fragments. Removal or exchange of the detergent and salts is tedious and is associated with significant losses of protein.

In this report we describe a method for the generation and isolation of peptide fragments for internal amino acid sequence analysis after separation of <100 pmol of the intact polypeptide chain by 1D or 2D PAGE. The method consists of the simultaneous electrophoretic transfer (electroblotting) of all separated proteins from the gel matrix onto nitrocellulose (12), detection of the proteins followed by *in situ* enzymatic cleavage of individual proteins on the nitrocellulose matrix, and reverse-phase HPLC separation of released peptide fragments. These peptide fragments are suitable for use in the gas-phase microsequenator without further manipulation. The general utility of the method is illustrated here by extensive sequence data from a variety of proteins prepared from 1D and 2D gel electrophoresis.

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Abbreviations: MBP, myelin basic protein; ARP, actin-related protein; 1D, one-dimensional; 2D, two-dimensional; PVP-40, polyvinylpyrrolidone, average M_r = 40,000.

EXPERIMENTAL PROCEDURES

Materials. For gel electrophoresis, standard commercial sources of chemicals were used. Nitrocellulose (0.45-μm pore size) was obtained from Schleicher & Schuell. The protein stains employed were Ponceau S (Sigma) or amido black 10B (Sigma). Polyvinylpyrrolidone, average M_r = 40,000 (PVP-40), was purchased from Sigma. L-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin and staphylococcal V-8 protease were obtained from Boehringer Mannheim. Polybrene was obtained from Sigma.

Gel Electrophoresis. Proteins were separated in $NaDodSO_4$/polyacrylamide gels with the dimensions 70 × 70 × 0.5 mm according to Laemmli (8) or by 2D PAGE (9). Shark myelin proteins were separated in polyacrylamide gels according to Thomas and Kornberg (13). High-purity gel chemicals were used and gels were polymerized extensively before use (11).

Electroblotting. Proteins were electroblotted in a Bio-Rad transblot system onto nitrocellulose as described (12) for 2 hr for 0.5-mm-thick gels. For proteins that were difficult to transfer, up to 0.005% $NaDodSO_4$ was added to the transfer buffer. After transfer, proteins were stained with amido black (14) or reversibly stained with Ponceau S using a modification of the method described (15) as follows. Nitrocellulose filters were immersed for 60 sec in a solution of 0.1% Ponceau S dye in 1% aqueous acetic acid. Excess stain was removed from the blot by gentle agitation for 1–2 min in 1% aqueous acetic acid. Protein-containing regions detected by either stain were cut out and transferred to Eppendorf tubes (1.5 ml), where Ponceau S-detected protein bands were destained by washing the filter with 200 μM NaOH for 1–2 min. Finally, the filter(s) was washed with distilled water and stored wet at −20°C, without extensive drying which can inhibit the efficient release of peptides by protease treatment.

***In Situ* Enzymatic Cleavage of Electroblotted Proteins.** Up to five bands ($NaDodSO_4$/PAGE) or 40 spots (2D gels) of destained nitrocellulose pieces containing the same protein were pooled in a single Eppendorf tube and incubated for 30 min at 37°C in 1.2 ml of 0.5% PVP-40 dissolved in 100 mM acetic acid in order to prevent adsorption of the protease to the nitrocellulose during digestion. Excess PVP-40 was removed by extensive washing with water (at least five changes). Because of the strong UV absorption of PVP-40, complete removal before HPLC analysis was essential. Nitrocellulose strips were then cut in small pieces of approximately 1 mm × 1 mm and put back into the same tube. The protein on the nitrocellulose pieces was digested as follows. (*i*) *Trypsin*: up to 300 μl of 100 mM Tris·HCl, pH 8.2/acetonitrile, 95:5 (vol/vol), at 37°C overnight. (*ii*) *V-8 protease*: 100 mM sodium phosphate, pH 7.8/acetonitrile, 95:5 (vol/vol), at 37°C overnight. Only the minimum volume of buffer necessary to completely immerse the nitrocellulose pieces was used. Enzyme-to-substrate ratio was kept at ≈1:20 (wt/wt). After digestion, the whole reaction mixture was frozen at −20°C or immediately loaded onto the HPLC column, after acidification.

Reverse-Phase HPLC of the Cleavage Fragments. Enzymatic cleavage fragments were separated on a narrow-bore (2.1-mm i.d.) reverse-phase HPLC system using a dual-syringe Brownlee micropump (16). The system was equipped with a Rheodyne model 7125 sample injector with a 500-μl loop. The analytical column used was a Brownlee Aquapore Bu-300, 2.1 × 100 mm, equipped with a guard cartridge filled with the same material. The following buffer system was used. Buffer A: 0.1% trifluoroacetic acid (Sequenal grade, Pierce) in water (double glass distilled). Buffer B: 0.08–0.095% trifluoroacetic acid in acetonitrile/H_2O, 70:30 (vol/vol). The optical densities of buffers A and B were matched at 215 nm by titrating the trifluoroacetic acid concentration in buffer B. Both buffers were degassed with a stream of helium. All experiments were carried out with columns at ambient temperature, at a flow rate of 100 μl/min. Gradients were run as indicated in Figs. 1–3. Peptides were detected by simultaneous monitoring at 215 nm, 260 nm, and 280 nm with a Waters 490 detector.

Fresh or thawed peptide-containing supernatant was acidified with 30 μl of 10% trifluoroacetic acid, mixed quickly in a Vortex, and centrifuged for 1 min in a Microfuge at high speed. The supernatant was removed and immediately injected into the HPLC unit. Buffer A was pumped through the column for 5 min at a flow rate of 200 μl/min before the flow was reduced to 100 μl/min and the gradient was started.

Peptide-containing fractions were collected manually into Eppendorf tubes based on the UV absorption at 215 nm. Collected fractions were frozen immediately. If this precaution was not taken the yield of recovered phenylthiohydantoin amino acids dropped significantly at each serine residue.

Peptide Sequence Analysis. Amino acid sequence analysis was performed on an automated Caltech gas-phase sequenator (5). Collected peptides were spotted onto glass fiber discs that had been coated with Polybrene and pretreated according to the following method so that precycling in the gas-phase sequenator was *not* necessary. A sheet of GF/F glass fiber paper (13-cm diameter) (Whatman) was soaked in a glass Petri dish for 2 min in a solution of 2 mg of Polybrene per ml of water (17) and dried in an oven at 50°C for 1 hr. Excess Polybrene and other contaminants were electrophoretically removed by exposing the sheet to an electric field (50 V) in a Transblot system (Bio-Rad) overnight at 4°C. The buffer tank contained a solution of 2 M acetic acid/methanol (Baker, HPLC grade), 1:1 (vol/vol), and several grams of mixed-bed ion-exchange resin (Amberlite MB-3, LKB). After electrophoretic purification, the glass fiber sheet was rinsed thoroughly in water, dried in an oven at 50°C, and stored in a clean glass Petri dish. Phenylthiohydantoin amino acids were identified essentially as described (18).

RESULTS AND DISCUSSION

Gel Electrophoresis, Electrophoretic Transfer, and Detection. The separation of components of even complex protein mixtures by 1D or 2D gel electrophoresis is perhaps the most commonly used protein analytical technique (8, 9). Similarly, the electrophoretic transfer of proteins from polyacrylamide gels onto nitrocellulose membranes is widely used for the detection and analysis of proteins with specific reagents (12). Although electroblotting onto nitrocellulose is generally efficient, the required presence of methanol in the transfer buffer tends to strip $NaDodSO_4$ from proteins, with the consequence that very basic and insoluble proteins transfer less efficiently. We have overcome this problem by adding small amounts of $NaDodSO_4$ (0.005%, wt/vol) to the transfer buffer to provide additional negative charges and help keep proteins in solution in cases where transfers were otherwise inefficient [e.g., myelin basic proteins (MBPs), pI > 9].

Transferred proteins were detected on the nitrocellulose membrane with Ponceau S or amido black. Although the sensitivity of Ponceau S staining does not match the sensitivity of amido black, <500 ng of protein per typical band can be detected. This sensitivity is sufficient to detect the amounts of proteins needed for internal sequence analysis. Staining with Ponceau S was preferred for the following reasons: (*i*) The method is fast. Blots are stained and destained in <5 min. (*ii*) Staining/destaining conditions are very mild. Proteins are not exposed to high concentrations of organic acids and solvents. (*iii*) Staining is reversible. Unlike other dyes, Ponceau S can be efficiently removed from the blotted proteins under very mild conditions (200 μM NaOH, 3–5 min) without eluting the protein from the nitrocellulose. In some cases, because of the high charge density, very basic

proteins such as MBPs cannot be completely destained. Neither residual Ponceau S nor amido black stain interferes with reverse-phase HPLC analysis of the peptide fragments from *in situ* digestion, since the dyes either remain on the nitrocellulose (amido black) or dissociate completely from the peptide fragments in HPLC buffer A (Ponceau S). The free dyes do not bind to C_4 columns and are washed through in the void volume.

Prevention of Protease Adsorption to Nitrocellulose. It was essential to pretreat the nitrocellulose before the protease digestion in order to prevent adsorption of the enzyme. Without such pretreatment, no cleavage was observed and the enzyme disappeared from the supernatant. We evaluated a number of nonprotein compounds [polyoxyethylene sorbitan monolaurate (Tween-20), polyethylene glycol (average M_r = 8000), PVP-40, Ficoll (approximate M_r = 400,000), and salmon sperm DNA] for their ability to bind to nitrocellulose and prevent adsorption of the protease during digestion. Nitrocellulose pieces were incubated with each reagent, reagents were washed away, and the saturated nitrocellulose pieces were incubated overnight at 37°C with ^{125}I-radiolabeled bovine serum albumin and washed. The number of radioactive cpm in the supernatant and on the membrane were determined. Bovine serum albumin was chosen as a model protein for these experiments because it is a relatively large and sticky protein. PVP-40 [0.5% (wt/vol) in 100 mM acetic acid, 30 min, 37°C] showed the highest saturating capacity (>90%) of all reagents tested. Furthermore, we found that the saturating capacity of PVP-40 was not pH dependent (pH range, 2–8) and did not increase with increasing concentration.

Efficiency of the Procedure. To quantitate the efficiency of the procedure, 10 μg of ^{125}I-radiolabeled α-lactalbumin was applied to 1-cm^2 pieces of nitrocellulose. The protein was stained with Ponceau S and destained, and the nitrocellulose pieces were saturated by treatment with 0.5% PVP-40 solution. Digestion with trypsin was carried out for 12 hr at 37°C and the resulting recovered peptide fragments were separated by HPLC. The efficiency of each step of the procedure was determined by γ-radiation counting. Although α-lactalbumin is a relatively small and nonsticky protein and represents an unfavorable case in terms of washout losses, the overall recovery of peptides amounted to ≈65% of the protein initially present on the nitrocellulose.

Two parameters proved to be critical for the high overall efficiency of the procedure. (*i*) Proteins are more strongly adsorbed to nitrocellulose at acidic than at neutral or basic pH. If the PVP-40 treatment was performed at acidic pH, very little protein was washed off during saturation. (*ii*) Hydrophilic interaction between peptide fragments and nitrocellulose can generally be reduced by the addition of organic solvent. Thus, 5% acetonitrile was included in the digestion buffer and proved to be sufficient for the effective release of the peptide fragments. No significant increase in the release of the peptide fragments was observed with up to 40% of acetonitrile in the digestion buffer, although this did not inhibit the proteolytic activity of trypsin (data not shown).

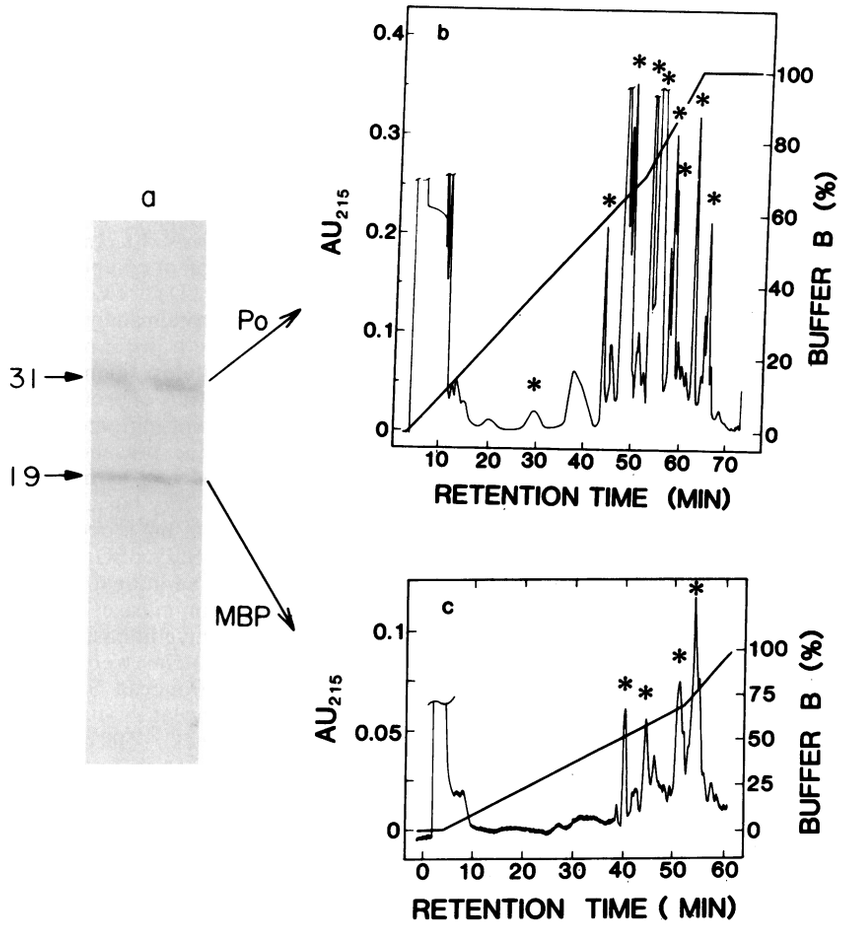

FIG. 1. Proteins of a shark brain myelin preparation. (*a*) Ponceau S-stained nitrocellulose blot of the proteins separated by 18% NaDodSO$_4$/PAGE (Kornberg gel) (13). Molecular weights are shown as $M_r \times 10^{-3}$. (*b*) HPLC map of peptides released after *in situ* tryptic digestion of P_o. (*c*) HPLC map of peptides released after *in situ* tryptic digestion of MBP. Asterisks (*) indicate peptides for which sequence analysis was carried out. AU, absorbance unit.

Peptide Mapping. Enzymatic peptide maps of the same protein digested either *in situ* on nitrocellulose blots or in solution are somewhat different, although most of the major peptides can be found in both preparations. Repetition of the process with multiple, electroblotted samples of α-lactalbumin showed that the digestion pattern is reproducible (data not shown). The differences in the peptide maps after enzymatic fragmentation *in situ* and in solution probably reflect differences in accessibility of enzymatic cleavage sites rather than differential release of peptides. The overall recovery, estimated from the relative peak areas in HPLC analyses of *in situ* and solution digests, correlates with the overall efficiency of 50–60% as calculated from radioactive labeling experiments. For typical amounts of proteins, the peptide cleavage fragments contained no contaminating peaks derived from the staining or PVP-40 saturation procedures or from the nitrocellulose itself. A protease blank reaction was always included to unambiguously identify substrate-derived peptides.

Application of the Method to Proteins Separated by NaDodSO$_4$/PAGE. In the context of our ongoing effort to understand the structure and function of the myelin sheath in the vertebrate nervous system (19), myelin proteins from the central nervous system of a shark (*Heterodontus*) were isolated and separated by NaDodSO$_4$/PAGE (13). After electrophoresis, the proteins were transferred onto nitrocellulose and stained with Ponceau S (Fig. 1*a*). The bands corresponding to MBP (≈5 μg) and to the myelin protein P$_o$ (≈12–14 μg) were cut out, destained, treated with PVP-40, and cleaved *in situ* with trypsin, and the resulting mixtures of peptide fragments were separated by reverse-phase HPLC. The peptide maps of shark P$_o$ and MBP are shown in Fig. 1 *b* and *c*, respectively. Selected peptides were applied to a gas-phase sequenator and their sequences were determined at the 20–50 pmol level. For both proteins sufficient internal sequence information was obtained for the synthesis of three independent oligodeoxynucleotide probes to screen shark brain cDNA libraries. The P$_o$ protein has an N terminus accessible to the Edman degradation, and therefore data for the synthesis of an oligonucleotide probe could be generated by conventional N-terminal sequence analysis (4). On the other hand, MBP is blocked at its N terminus, so that sequence information could only be obtained from cleavage fragments of the protein. MBP is a very basic protein (pI > 9). Although this protein could not be completely destained after Ponceau S treatment, no contaminating peaks interfered with peptide separation or detection (Fig. 1*c*). The large number of basic residues in MBP led to the generation of a number of very small tryptic cleavage fragments that did not bind to the C$_4$ column, explaining the relatively small number of peptides recovered after HPLC separation.

Application of the Method to Proteins Separated by 2D PAGE. We have applied the nitrocellulose blot/*in situ* digestion method to a number of proteins isolated from 2D polyacrylamide gels of whole-cell lysates. The sequence data were used to confirm the identity of spots, to reveal homologies to known proteins, or to synthesize oligodeoxynucleotide probes for isolation of the gene. The results obtained with two proteins are illustrated in Fig. 2. As 2D gels have a limited loading capacity, multiple spots (20–40) (≈10–15 μg) of the same protein species were accumulated and simultaneously processed. To reduce the number of second-dimension and electroblotting experiments, the relevant regions of multiple (up to five) first-dimension gels were aligned onto a single second-dimension gel as described (21). Corresponding spots from the repeated pattern on the nitrocellulose blots were cut out and simultaneously subjected to *in situ* digestion. Fig. 2 shows peptide maps of two proteins from a human lymphoblastoid cell line: actin-related protein (ARP), a protein corresponding to p24 in a 2D gel protein catalog (22) (Fig. 2*b*), and the β subunit of mitochondrial F$_1$-ATPase (Fig. 2*c*). A total of 107 residues of sequence data were generated from nine peptides from the F$_1$-ATPase digest and aligned with the known sequence of the bovine protein (23). The high degree of homology (>90%) confirmed the identity of the human lymphoblastoid protein in the 2D gel. Similar internal sequence data were obtained for ARP, the other protein example in Fig. 2. Computer-assisted searches of sequence data bases showed that ARP, which is known to be an abundant nucleus-encoded protein subsequently transported

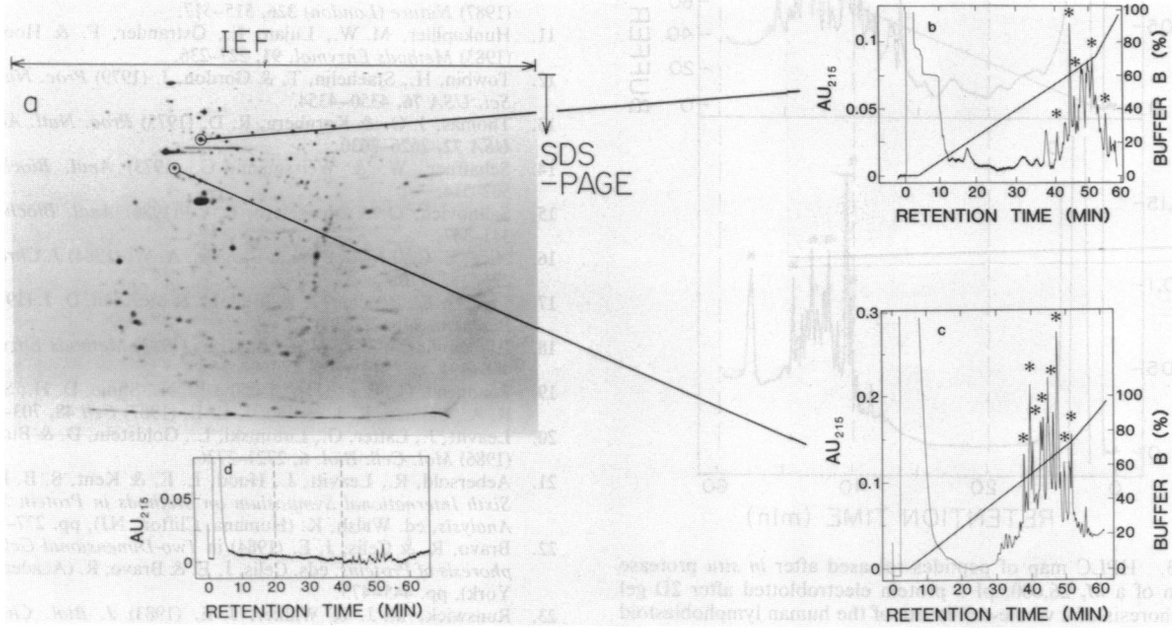

FIG. 2. Proteins from a whole-cell lysate of the human lymphoblastoid cell line CCRF-CEM (ATCC no. CCL 119). (*a*) 2D [isoelectric focusing (IEF)/NaDodSO$_4$/PAGE] separation of proteins metabolically labeled with [^{35}S]methionine (20). Proteins used as examples are circled. (*b*) HPLC map of peptides released after *in situ* tryptic digestion of the protein ARP after electroblotting onto nitrocellulose. (*c*) HPLC map of peptides released after *in situ* tryptic digestion of the β-subunit of mitochondrial F$_1$-ATPase after electroblotting onto nitrocellulose. (*d*) Blank digest with trypsin. Asterisks (*) indicate peptides for which sequence analysis was carried out. AU, absorbance unit.

into mitochondria, does not show significant homology to any known protein.

CONCLUSIONS

We have described a method for obtaining extensive internal sequence information from microgram amounts of protein. The nitrocellulose blot/*in situ* digestion method offers a number of advantages over previous methods used to obtain internal sequence information from proteins. It employs the most highly resolving and generally applicable protein separation methods, 1D and 2D PAGE, and allows the parallel isolation, in a single operation, of multiple proteins in a form suitable for internal sequence analysis even if they are blocked at the N terminus. We estimate that, even with existing levels of protein sequencing sensitivity, on the order of 100 proteins from the 2D gel separation of a whole-cell lysate (20) are present in sufficient amounts to yield extensive amino acid sequence information using the strategies outlined in this report.

The method is compatible with proteolytic enzymes of different specificity (Fig. 3), offering the possibility of obtaining long stretches of overlapping sequence information, or even the complete sequence from microgram amounts of a given protein. Typically, however, it will be extremely useful to obtain multiple independent stretches of internal sequence data to assist in cloning the corresponding gene. An additional important use for extensive amino acid sequence data is to establish homology relationships with known proteins.

A further consequence of the methods reported here is that the preparation of protein samples is no longer the limiting step in protein sequence analysis. An optimum strategy for protein isolation no longer requires a tedious multistep purification procedure, often resulting in low recoveries, but rather the preparation of a protein mixture in which the component of interest is enriched with minimal losses and identified. Many biomedically interesting proteins are available only in limited amounts that require such efficient isolation procedures. The goal of this study has been to develop a generally applicable technique for the high-yield preparation of peptide fragments for internal amino acid sequence analysis of proteins. The method described above provides a solution to this problem and should find wide application. In addition to protein sequencing, the method is useful for analytical peptide mapping and for defining secondary structure modifications (such as phosphorylation) (24).

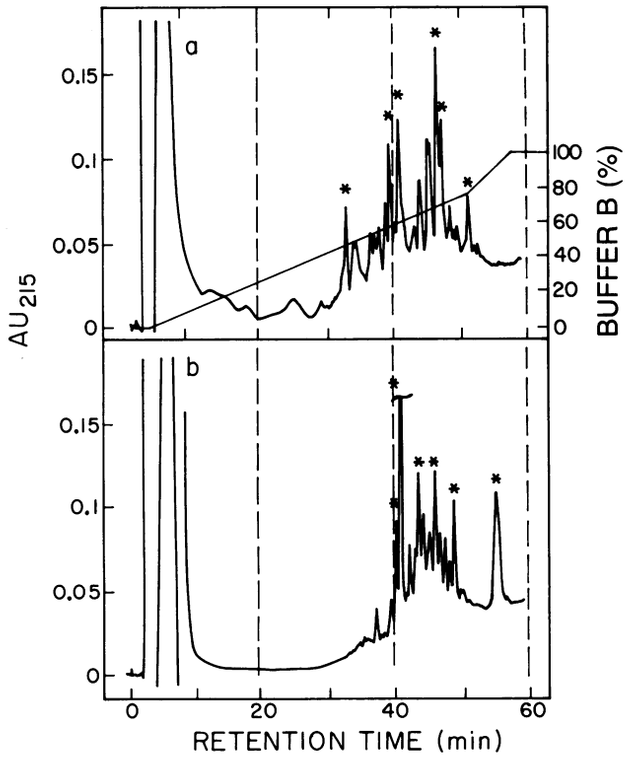

FIG. 3. HPLC map of peptides released after *in situ* protease digestion of a M_r 26,000, pI 5 protein electroblotted after 2D gel electrophoresis of a whole-cell lysate of the human lymphoblastoid cell line CCRF-CEM. (*a*) Trypsin digest. (*b*) *Staphylococcus aureas* V-8 protease digest. Peaks marked with an asterisk (*) were subjected to sequence analysis. AU, absorbance unit.

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