Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 7008–7012, September 1988
Neurobiology

Sequencing of proteins from two-dimensional gels by using *in situ* digestion and transfer of peptides to polyvinylidene difluoride membranes: Application to proteins associated with sensitization in *Aplysia*

(long-term sensitization/long-term memory/protein sequencing)

T. E. KENNEDY, M. A. GAWINOWICZ, A. BARZILAI, E. R. KANDEL, AND J. D. SWEATT

Howard Hughes Medical Institute and Center for Neurobiology and Behavior, College of Physicians and Surgeons of Columbia University, 722 West 168th Street, New York, NY 10032

Contributed by E. R. Kandel, May 31, 1988

ABSTRACT **We have developed a method for obtaining partial internal amino acid sequence data from proteins isolated directly from preparative two-dimensional polyacrylamide gels. Proteins from a crude cell homogenate are separated using preparative two-dimensional polyacrylamide gel electrophoresis. Then, the gel is stained with Coomassie blue and the protein spots of interest are cut out. The *in situ* protein is digested with *Staphylococcus aureus* V8 protease in a second polyacrylamide gel and the peptides are separated by one-dimensional polyacrylamide gel electrophoresis. The peptides are then electroblotted onto a polyvinylidene difluoride membrane, visualized using Coomassie blue, cut out, and sequenced using an automated gas phase sequencer. Using this method, we have obtained amino acid sequence data for two proteins that are altered after long-term sensitization: actin and *Aplysia* protein 407. In addition, we have obtained amino acid sequence data for rat protein 425, a protein that appears to be homologous to *Aplysia* protein 407.**

High-resolution two-dimensional polyacrylamide gels are commonly used as an analytical approach to characterize proteins. The power of this approach could be enhanced if this method for detecting and separating proteins were combined with a means of obtaining sequence data from individual proteins detected on these gels. We report here the development of a method for obtaining amino acid sequence information from proteins isolated on preparative one- or two-dimensional polyacrylamide gels. The method is a combination of previously used analytical and preparative procedures (1–4). The protein to be sequenced is first isolated by excising it from a Coomassie blue-stained one- or two-dimensional gel. The protein is then digested *in situ* with *Staphylococcus aureus* V8 protease, and the resulting peptides are electrophoresed in a second polyacrylamide gel (3). These peptides are then transferred to a polyvinylidene difluoride membrane (Immobilon, Millipore) (4), visualized by Coomassie blue staining, cut out, and sequenced using an automated gas-phase sequencer.

The method has four advantages over previously used procedures. First, it is technically straightforward and inexpensive, using reagents and nonspecialized equipment available in most biochemistry laboratories. Second, when combined with automated gas-phase sequencing, it is sensitive and generates amino acid sequence information from small amounts of protein, 1–10 μg generally being sufficient. Third, an internal peptide sequence can usually be obtained. Thus, the method provides a convenient, practical means to overcome the limitations in sequencing proteins with blocked amino termini. Finally, the isolation of proteins from two-dimensional gels allows one to purify a particular protein in just a few steps, starting from a crude cell extract and proceeding to amino acid sequence.

Although generally applicable, we specifically developed this method to extend, to the molecular level, our analysis of changes in protein phosphorylation and protein synthesis induced by short- and long-term sensitization of the gill-and-siphon-withdrawal reflex in *Aplysia*. We initially worked with an abundant protein, actin, which is a substrate for the cAMP-dependent protein kinase in *Aplysia* sensory neurons and whose phosphorylation increases with sensitization of these neurons. Because the applicability of this method also extends to less abundant proteins, we next worked with *Aplysia* protein 407 (5), whose net rate of synthesis is increased with long-term sensitization. We obtained internal, partial amino acid sequence data on *Aplysia* protein 407. The method should be generally applicable to other situations where it is desirable to obtain amino acid sequence information on proteins of biological interest identified on two-dimensional gels.

METHODS

Purification of *Aplysia* Actin. *Aplysia californica* (100–200 g) were obtained from Sea Life Supply (Sand City, CA). Animals were anesthetized by injection of approximately half their body weight of isotonic $MgCl_2$ solution. The total central nervous system (buccal, cerebral, pedal, pleural, and abdominal ganglia with surrounding connective tissue) was then dissected out and ground in a glass/glass tissue grinder in 20 mM Tris·HCl, pH 7.6/10 mM EGTA/5 mM EDTA/1 mM $MgCl_2$ containing leupeptin at 10 μg/ml, aprotinin at 10 μg/ml, 1 mM benzamidine, 1 mM dithiothreitol, and 0.6 mM phenylmethylsulfonylfluoride (homogenization buffer). The resulting suspension was then homogenized (five strokes using a motor-driven pestle) in a glass/Teflon homogenizer. The homogenate was centrifuged at 50,000 × *g* in a Beckman ultracentrifuge (type 70.1 Ti rotor) for 1 hr at 4°C to remove insoluble material.

Actin was partially purified from the resulting supernatant, using the first two steps of the procedure described by Gordon *et al.* (6). Briefly, the 50,000 × *g* supernatant was loaded onto a 3-ml column of DEAE-Sephacel (Pharmacia) preequilibrated with homogenization buffer. After the supernatant was loaded, the DEAE column was washed sequentially with 3 volumes of homogenization buffer, followed by 3 volumes of homogenization buffer containing 125 mM KCl, and actin was eluted with 1.5 column volumes of homogenization buffer containing 225 mM KCl. The eluate was concentrated by centrifugation using a Centricon 30 (Amicon)

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

device. The concentrated DEAE eluate was loaded onto a Pharmacia Superose 12 FPLC gel filtration column. The column was run in homogenization buffer at a flow rate of 0.3 ml/min using a Pharmacia FPLC pump, and the major actin-containing fractions were collected and pooled. As a final purification step, the actin in this pool was purified using preparative electrophoresis (see Fig. 1) on a 10% polyacrylamide gel (1).

Purification of *Aplysia* Protein 407. Total *Aplysia* central nervous system was ground in a glass/glass tissue grinder in 0.3% $NaDodSO_4$/5% 2-mercaptoethanol/20 mM Tris·HCl, pH 8.0, DNase 1 at 0.1 mg/ml and RNase A at 50 μg/ml (sample buffer) and then homogenized in a glass/Teflon homogenizer. The tissue homogenate was briefly spun in a microcentrifuge at 16,000 × *g* to remove large particulate material, and the supernatant was stored at −20°C.

Aplysia protein 407 was isolated using preparative two-dimensional gel electrophoresis essentially as described by O'Farrell (2). Two-dimensional electrophoresis for purification of *Aplysia* protein 407 was performed at Protein Databases (Huntington Station, NY) using their commercially available two-dimensional preparative gel electrophoresis system. Five hundred micrograms of protein homogenate from the total central nervous system of two or three animals was loaded per first dimension [2-mm diameter, Resolyte 4–8 ampholines (BDH)] of the two-dimensional preparative gels (24 cm, 24 cm, 2 mm). After electrophoresis, the gels were stained with Coomassie blue [0.5% in 50% methanol/10% acetic acid (vol/vol)] and destained in 30% methanol/10% acetic acid (vol/vol). Approximately 200 proteins were visible on each two-dimensional preparative gel stained with Coomassie blue. Protein 407 was then identified by comparison of its migration on these gels with the pattern seen on the fluorograms and silver-stained gels used previously to identify spot 407 as a protein whose [^{35}S]methionine incorporation changes with long-term sensitization training in *Aplysia* (5). Individual Coomassie blue-stained protein spots corresponding to spot 407 were then cut out and stored in a minimal volume of 50% methanol at −20°C.

Protease V8 Digestion. Material isolated from preparative two-dimensional gels was digested *in situ* using *Staphylococcus aureus* V8 protease essentially as described by Cleveland *et al.* (3). The material cut from the preparative gels was soaked in deionized water for 15 min to reduce the content of methanol and acetic acid present after destaining. The gel pieces were then loaded into a large well (10 mm × 29 mm × 1.5 mm) of a second single-dimension 15% acrylamide gel (200 mm × 160 mm × 1.5 mm) either as an acrylamide cube or as a slurry of material ground in an Eppendorf tube in the presence of 20% glycerol (vol/vol)/0.05 M Tris, pH 6.8/0.2% bromophenol blue/0.1% $NaDodSO_4$ containing V8 enzyme at 10 μg/ml (4.5×10^{-2} unit/ml) (V8 protease buffer). After the material was loaded, the remainder of the sample well was filled with V8 enzyme in buffer. Typically we used a 1:1 (vol/vol) ratio of gel slice to protease buffer (approximately 100 μl, 1 μg of enzyme). Grinding the gel cubes using an Eppendorf tube micropestle (Kontes) appeared to improve enzymatic digestion, perhaps by giving both the V8 enzyme and the buffer $NaDodSO_4$ better access to the protein contained in the acrylamide matrix. We have successfully used *Staphylococcus aureus* V8 protease (endoprotease Glu-C, EC 3.4.21.19) from Sigma, Calbiochem, or Boehringer Mannheim.

To scavenge free radicals remaining in the gel and to minimize the possibility of modification of reactive amino acid residues or of amino termini of the peptides during electrophoresis, 0.002% thioglycolic acid was added to the running buffer in the upper chamber of the electrophoresis apparatus.

The acrylamide/protein/enzyme mixture was allowed to incubate in the well for 15 min and then run to the stacker/separating gel interface at a low constant voltage (50–70 V). At this point the current was turned off, for approximately one-half hour, to allow the enzyme to digest the protein *in situ*. The amount of digestion could be altered by varying the length of time the focused protein/enzyme mixture was held at the interface or by altering the substrate/enzyme ratio. In this way the relative amounts of various peptides could be changed to obtain internal sequence data from different parts of the protein (see Fig. 1). After digestion, electrophoresis was completed, typically at 200–250 V, using constant voltage.

Electroblotting. Peptides were blotted onto a polyvinylidene difluoride membrane, which was then stained with Coomassie blue essentially as described by Matsudaira (4). Briefly, the gels were soaked for 10 min in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid/10% methanol, pH 11.0 (transfer buffer). Polyvinylidene difluoride membranes were cut to size and wetted with 100% methanol, then rinsed with transfer buffer. Electroblotting was carried out in a Hoefer TE series Transphor Electrophoresis unit at 1.5 A for 30 min (Hoefer, San Francisco). After transfer, the blots were washed with deionized water for 5 min, stained for 5 min with 0.1% Coomassie blue in 50% methanol (vol/vol), and then destained with 50% methanol/10% acetic acid (vol/vol). After washing with deionized water, the blots were dried briefly in a vacuum oven (70°C, −80 μPa for about 5 min), and the peptide bands were cut out and stored dry in Eppendorf tubes at −20°C.

Protein Sequencing. Sequencing was carried out on an Applied Biosystems (Foster City, CA) 470A gas-phase sequencer equipped with an on-line analyzer for phenylthiohydantoin-derivatized amino acids. A trifluoroacetic acid-activated glass fiber filter was coated with 3 mg of Polybrene and precycled in the sequencer to remove contaminants. The peptide band was placed so that it was positioned on top of the glass fiber filter in the assembled sample cartridge. The peptide was sequenced using the standard 03RPTH program.

RESULTS

To develop this method, we first worked with actin isolated from *Aplysia* central nervous system. Actin offers the advantages that it is an abundant protein (approximately 10% of total protein in the *Aplysia* total central nervous system) and has a very high degree of sequence homology across species (7). In addition, actin is of specific interest to us because it is a protein whose phosphorylation increases in response to serotonin and cAMP application in *Aplysia* sensory neurons (J.D.S. and E.R.K., unpublished data). Actin typically has a blocked amino terminus (8), making it especially suited to the development of an approach designed to obtain internal amino acid sequence on blocked proteins.

Sequencing of *Aplysia* Actin. The typical Coomassie blue staining pattern obtained after the final preparative electrophoresis step of the actin purification protocol is illustrated in Fig. 1*A*. The actin-containing band (arrow) was cut out of the preparative gel, the protein was digested *in situ*, and the peptides were electroblotted to a polyvinylidene difluoride membrane as described in *Methods*. Coomassie blue staining patterns obtained after blotting the digested actin are illustrated in Fig. 1 *B* and *C*. As mentioned in *Methods*, varying the substrate/V8 protease ratio altered the digestion of the substrate. The pattern obtained at a substrate/V8 ratio (wt/wt) of 50:1 is illustrated in Fig. 1*B*; that obtained at a ratio of 10:1 is illustrated in Fig. 1*C*, which also shows the amino acid sequence obtained on sequencing each peptide. In typical preparations we obtained ≈150 pmol each of several

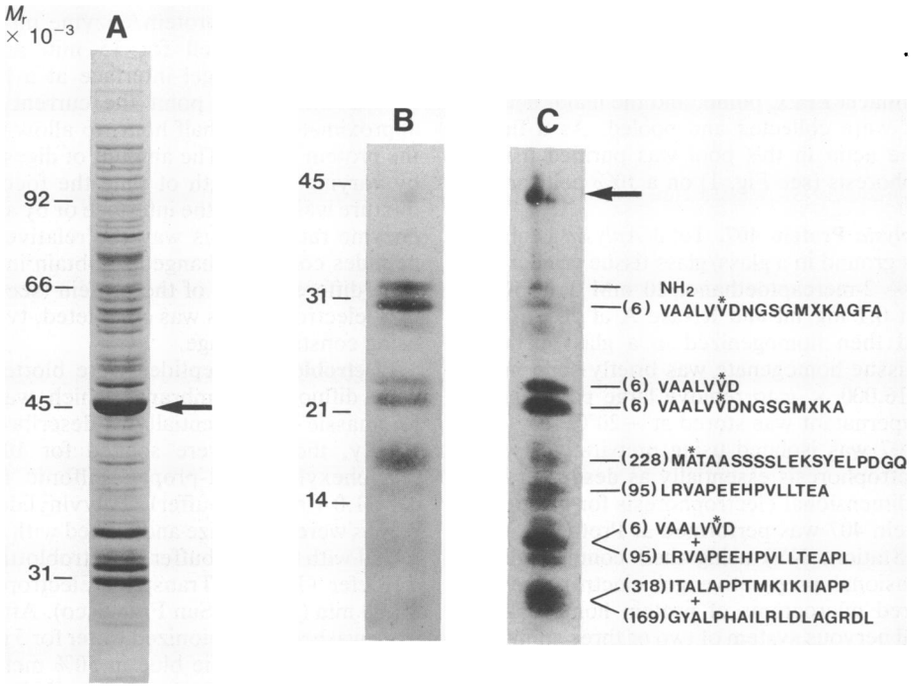

FIG. 1. (*A*) Coomassie blue staining pattern of partially purified actin. Approximately 50 μg of protein from an actin-containing gel-filtration fraction was loaded onto a 10% polyacrylamide gel. The actin-containing band (arrow) was cut out and subjected to V8 protease digestion, electrophoresis, and transfer as described in *Methods*. (*B* and *C*) Coomassie blue staining patterns of actin fragments following V8 protease digestion at actin/V8 ratios of 50:1 (*B*) and 10:1 (*C*). Amino acid sequencing of each peptide gave the results shown to the right of *C*. The arrow in *C* indicates undigested actin. Numbers in parentheses are beginning residue numbers as determined by alignment with the *Strongylocentrotus purpuratus* actin sequence (9). Sequence differences between the sequence shown and that reported for *Strongylocentrotus purpuratus* actin are marked with asterisks. NH_2, presumed amino terminus of actin (this peptide gave no sequence). The V8 protease present in the digestion mixture typically was present on the membrane after transfer and showed up as a faint Coomassie blue staining band at M_r 30,000. The use of blank lanes containing V8 protease allowed easy identification of this protein, and the accidental sequencing of this protein can be eliminated as a possibility by comparison of the derived amino acid sequences to the published sequence for V8 protease (10).

peptides, starting with ≈10 μg of actin isolated from the final preparative electrophoresis step.

Of 52 residues of amino acid sequence obtained for *Aplysia* actin, only 2, marked with asterisks (*) in Fig. 1, differ from the reported sequence for *Strongylocentrotus purpuratus* (sea urchin) actin (11). These data are in good agreement with a number of previous reports of a high degree of sequence conservation for actin from a variety of tissue sources and species. Although multiple isoforms of actin are known to exist (12), these different actins generally have a high degree of sequence homology. Thus, we might in fact have two or more species of actin in our final preparation but, because of sequence homology, be able to detect only one amino acid sequence.

We next applied this digestion and sequencing protocol to actin cut from two-dimensional gels run on a whole cell homogenate from the *Aplysia* total central nervous system. We obtained amino acid sequence data from actin isolated from the two-dimensional gels identical to those obtained from the purified material (data not shown). Thus, using a whole cell homogenate as starting material, we were able to obtain microsequence data for actin purified by means of a one-step preparative two-dimensional gel procedure.

Sequencing of Spot 407. The success of this two-dimensional gel purification approach with an abundant protein encouraged us to apply it to less-abundant proteins. Therefore, we next focused on a protein that we had earlier identified as one in which the rate of synthesis changes during the maintenance phase of long-term sensitization in *Aplysia* (5). Protein 407 from the abdominal ganglion has been shown by Castellucci *et al.* (5) to have an increased rate of incorporation of [^{35}S]methionine after long-term sensitization of the gill-and-siphon-withdrawal reflex. Protein 407 accounts for ≈0.1% of *Aplysia* central nervous system protein and can be identified on preparative two-dimensional gels of *Aplysia* central nervous system lysate. We cut the Coomassie blue-stained spot from eight preparative gels and digested the protein *in situ* with *Staphylococcus aureus* V8 enzyme. Each spot cut from the gel contained ≈0.1 μg of protein 407 for a total of ≈1 μg of material digested (≈20 pmol of a protein of M_r 50,000). The digest was then electroblotted and stained, producing a blot containing a single abundant peptide of M_r ≈20,000 (Fig. 2*A*). Sequencing of this peptide gave the results shown in Fig. 2*A*.

Using a two-dimensional gel protein database established at Protein Databases, we observed in silver-stained two-dimensional gels of a crude cell homogenate of cultured Wt2 rat embryo fibroblasts a protein that comigrated with *Aplysia* protein 407, rat protein 425. We cut this protein from eight preparative gels loaded with rat fibroblast whole cell lysate, digested it *in situ* with *Staphylococcus aureus* V8 protease, and blotted the peptides as described in *Methods*. The V8 digest of the rat material produced a peptide pattern similar to that observed for the *Aplysia* protein (Fig. 2*B*). We sequenced the major band of the rat material that corresponded in molecular weight to the major band of the *Aplysia* protein 407 V8 digest. *Aplysia* protein 407 and rat protein 425 show 7 of 11 amino acids to be identical, suggesting that they are homologous proteins. Whether rat protein 425 is expressed in rat brain and whether its expression also changes during certain forms of learning and memory remain to be determined.

The results obtained with *Aplysia* protein 407 and rat fibroblast protein 425 demonstrate the applicability of this

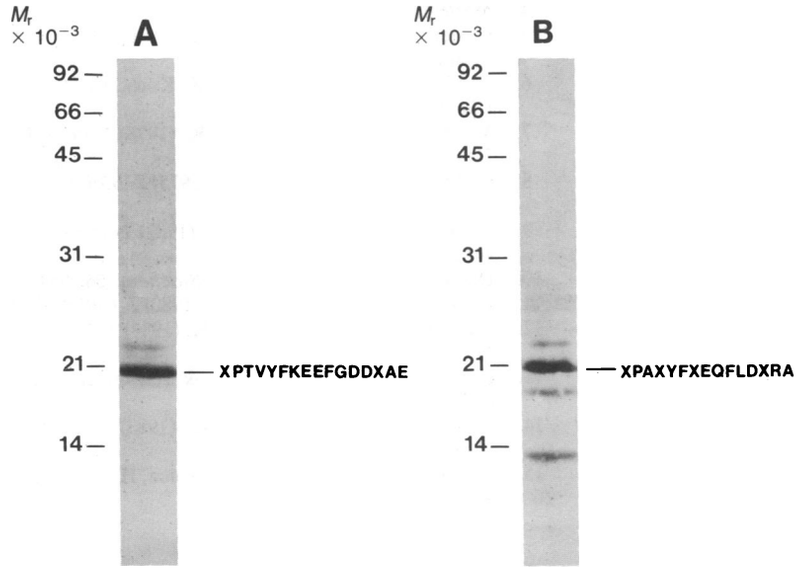

FIG. 2. (*A*) Coomassie blue-staining pattern obtained after *in situ* digestion and electroblotting of ≈1 μg (20 pmol) of *Aplysia* protein 407 cut from eight two-dimensional preparative gels. The amino acid sequence of the major band (M_r ≈ 20,000) is indicated. Approximately 5 pmol of amino acid derivative were obtained for each cycle of sequencing. (*B*) Coomassie blue-staining pattern obtained after *in situ* digestion and electroblotting of the comigrating rat protein isolated from eight preparative two-dimensional gels. The amino acid sequence of the rat peptide band corresponding to the major *Aplysia* protein 407 (M_r ≈ 20,000) is indicated.

method to low-abundance proteins. We estimate that pooling the material from about 10 preparative two-dimensional gels should generate sufficient material for sequencing the 200 most-abundant proteins in a crude cell homogenate preparation. A useful general rule is that sequence may be obtained from proteins visible as Coomassie blue-stained protein spots when material from several gels is combined.

One limitation to the present method is that integral membrane proteins do not transfer well from the first, preparative gel slice into the second, protease-digestion gel. In preliminary experiments with Kevin Karl, we have used reducible, disulfide bond-containing crosslinkers in the first gel to overcome this limitation.

DISCUSSION

Previous work in this laboratory has focused on the biology of learning in the marine snail *Aplysia californica*. As part of an ongoing investigation into the cellular biochemistry of nervous function in *Aplysia*, we set out to develop methods that would allow us to obtain protein sequence information on picomolar amounts of material isolated from two-dimensional gels. To develop this method, we chose initially to work with actin, as we had identified this protein as one in which phosphorylation increases in response to serotonin in sensory neurons (J.D.S. and E.R.K., unpublished data). Actin was desirable to work with initially, as it is an abundant protein in most cell types and has a high level of sequence homology across species. Using the preparative technique described in this paper, we obtained 52 residues of amino acid sequence from nine *Aplysia* actin peptide fragments. *Aplysia* actin has been previously purified and immunologically characterized (11, 13, 14); however, to our knowledge, this is the first reported sequence information for an *Aplysia* actin. These data show that *Aplysia* actin is ≈95% homologous to *Strongylocentrotus purpuratus* (sea urchin) actin (9).

The data obtained in sequencing actin illustrate several desirable features of this method for protein sequencing. Earlier and unsuccessful attempts to obtain amino-terminal sequence information on pure, undigested actin suggested that the protein has a blocked amino terminus (data not shown), as is typical for this protein. However, we were able to overcome this limitation and generate internal amino acid sequence. In addition, multiple stretches of internal amino acid sequence were obtained, which is useful for making a more reliable identification of the protein. The method is also potentially useful for determining sites of posttranslational modification. For example, phosphorylation sites present on particular peptides could be identified by labeling the protein with $^{32}PO_4$.

With this method, we also obtained 14 residues of amino acid sequence from one peptide fragment of a protein, *Aplysia* protein 407, whose expression is increased after long-term sensitization. Using the Protein Identification Resource of the National Biomedical Research Foundation, we searched for potential sequence homologies to *Aplysia* protein 407 and found that the best match equaled 54% identity at the amino acid level. However, using the Protein Database two-dimensional gel protein data base, we observed the comigration of *Aplysia* protein 407 with a protein present in a cultured Wt2 rat fibroblast extract, rat protein 425. *Staphylococcus* V8 protease mapping of this protein yielded a peptide pattern similar to that of *Aplysia* protein 407 (see Fig. 2). Eleven residues of amino acid sequence were determined for the rat protein, of which seven are identical to *Aplysia* protein 407 (64% amino acid identity). A search of the protein sequence data base showed that the best match to rat protein 425 was 45% amino acid identity. Thus, *Aplysia* protein 407 showed greater homology to rat protein 425 than to any other protein in either protein sequence data base. These data, coupled with the observation of similar molecular weights, isoelectric points, and V8 peptide maps, suggest that *Aplysia* protein 407 and rat fibroblast protein 425 are homologous.

Amino acid sequence information can serve as a starting point for further pursuits. For example, it allows the construction of oligonucleotide probes that may be used to screen cDNA libraries to isolate, clone, and characterize the corresponding mRNA. Obtaining a cDNA clone, in addition to providing further sequence information, allows quantitative analysis of message expression and may be used to address cellular localization using *in situ* hybridization. Also, peptides can be synthesized, according to amino acid sequence data, and antibodies can be raised against them. These may be used to examine the expression of the protein, as well as its cellular and subcellular localization.

The method of peptide purification that we have developed has made it possible to move readily from the level of analytical two-dimensional gel analysis to preparative two-dimensional gels and protein sequencing. This technique should be widely applicable as a means of characterizing a protein of interest that has been identified on a one- or two-dimensional gel. Using simple equipment and inexpensive reagents, the method makes it possible to generate

several sequenceable peptides in good yield from a single protein, providing multiple stretches of internal sequence data.

Note Added in Proof. Brown *et al.* (15) have used a similar approach to obtain the amino acid sequence from immunoaffinity-purified material digested with *Staphylococcus aureus* V8 protease and blotted to polyvinylidene diflouoride.

We thank M. Crouse and G. Berube from Protein Databases Inc. for their technical assistance and expertise throughout this project. We also thank K. Hilten and L. Katz, who prepared the illustrations, and H. Ayers and A. Krawetz, who typed the manuscript. We acknowledge support by the Howard Hughes Medical Institute and a National Science and Engineering Research Council of Canada postgraduate scholarship to T.E.K.

1. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
2. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250,** 4007–4021.
3. Cleveland, D. W., Fischer, S. G., Kirschner, M. W. & Laemmli, U. K. (1977) *J. Biol. Chem.* **252,** 1102–1106.
4. Matsudaira, P. (1987) *J. Biol. Chem.* **262,** 10035–10038.
5. Castellucci, V. F., Kennedy, T. E., Kandel, E. R. & Goelet, P. (1988) *Neuron* **1,** 321–328.
6. Gordon, D. J., Eisenberg, E. & Korn, E. D. (1976) *J. Biol. Chem.* **251,** 4778–4786.
7. Vandekerckhove, J. & Weber, K. (1978) *Nature (London)* **276,** 720–721.
8. Collins, J. H. & Elzinga, M. J. (1975) *J. Biol. Chem.* **250,** 5915–5920.
9. Cooper, A. D. & Crain, W. R. (1982) *Nucleic Acids Res.* **10,** 4081–4092.
10. Drapeau, G. R. (1978) *Can. J. Biochem.* **56,** 534–544.
11. Lubit, B. W. & Schwartz, J. H. (1980) *J. Cell Biol.* **86,** 891–897.
12. Vandekerckhove, J. & Weber, K. (1984) *J. Mol. Biol.* **179,** 391–413.
13. Harris, D. A., Sherbary, A. A. & Schwartz, J. H. (1984) *Biol. Bull.* **166,** 482–493.
14. Lubit, B. W. & Schwartz, J. H. (1983) *J. Histochem. Cytochem.* **31,** 728–736.
15. Brown, T. A., Prahl, J. M. & DeLuca, H. F. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 2454–2458.