Internal Amino Acid Sequence Analysis of Proteins Separated by Gel Electrophoresis after Tryptic Digestion in Polyacrylamide Matrix

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Key Words

Electrophoresis
Polyacrylamide gel
Proteins
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Summary

A method is described for obtaining peptide fragments for sequence analysis from microquantities of proteins separated by 1- or 2-dimensional polyacrylamide gel electrophoresis. After separation by electrophoresis, the proteins were stained with Coomassie Blue and excised. Proteolytic digestion with trypsin was performed directly in the polyacrylamide matrix. The resulting peptide fragments were eluted, separated by reversed phase HPLC, collected and sequenced in a gas phase sequencer. Excellent peptide recoveries allowed 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 sequences that can be used to generate oligonucleotide probes for molecular cloning, to design synthetic peptides for inducing antibodies, and to search sequence databases for related proteins.

Introduction

For analysis of complex mixtures of proteins SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is commonly used because of its high resolution and good reproducibility [1]. Using this technique a number of markers for polymorphism, genetic diseases or tumors can be identified. However, the major drawback is that the proteins recognized due to their correlation with the problem investigated could almost never be further analysed. Except for a very limited number of immunologically characterized proteins the nature and identity of these markers remains unclear, mainly because i) the protein chemical methods were too insensitive to deal with the amounts present in a 2-D polyacrylamide gel and ii) the proteins separated by PAGE are embedded in a matrix incompatible with protein chemical methods such as amino acid sequence analysis and amino acid composition analysis.

In the last several years progress in the methods of amino acid sequence analysis and amino acid analysis has made it possible to handle low microgram quantities of proteins and, more importantly, methods have become available to immobilize proteins from a polyacrylamide matrix on to chemically inert membranes by electroblotting [2–5] (see Fig. 1). These immobilized proteins can be subjected directly to protein sequence analysis and amino acid composition analysis. Enough information can be obtained from even a single spot from a 2-D gel to allow identification of the proteins stored in sequence databases [6, 12].

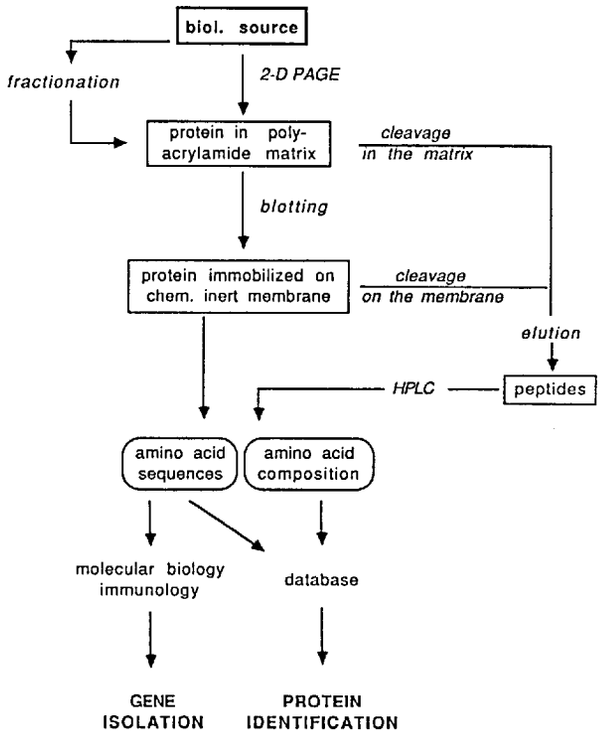

Fig. 1

Strategies to obtain amino acid sequence information from electrophoretically separated proteins.

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Furthermore, the amino acid sequence information can be used to search for homologies with other known proteins, to construct oligonucleotide probes for gene isolation, or to construct synthetic peptides which can then be used to induce monospecific antibodies against the protein.

However, many proteins are not susceptible to Edman degradation due to biological or artifactual blocking of the α-amino group. Therefore, amino acid sequence information from the interior of the protein is necessary. For the generation of internal peptides after electrophoresis two alternatives are possible: cleavage of the protein either in the polyacrylamide matrix or on the membrane after electrophoretic transfer (see Fig. 1).

In this report the strategy and the methods to obtain internal amino acid sequences are described and the advantages and limitations of the various methods are discussed.

Methods

SDS Polyacrylamide Electrophoresis

The samples were prepared and separated by 1D PAGE according to Laemmli [7] and by 2D PAGE according to O'Farrell [8]. If necessary proteins were concentrated and desalted using Centricon microconcentrators (Amicon) prior to electrophoresis. It was important to keep a maximum protein to gel ratio. The polyacrylamide concentration was kept as low as possible to obtain a good separation. After electrophoresis the proteins were stained briefly with Coomassie Blue and destained for several hours in 10% acetic acid/30% methanol.

Preparation of the Protein Spots/Bands for Enzymatic Digestion

Protein-containing bands were excised precisely with a scalpel to minimize the amount of polyacrylamide matrix. The gel pieces were incubated for at least 24h in double distilled water with frequent water changes. Protein bands from 1D gels were diced but not ground. After the extensive wash the gel pieces were lyophilized. It was found to be important not to dry the gel pieces completely; some moisture should be retained. Blank gel pieces served as a control for autodigestion of trypsin and were run in parallel with the protein spots of interest.

In situ Tryptic Digestion in the Polyacrylamide Matrix

The dry gel pieces were incubated for 5h at 37 °C in 100 mM ammonium-hydrogen carbonate, pH 8.5, 0.5 mM calcium chloride and trypsin (trypsin: protein approximately 1 : 10). A minimum reaction volume, sufficient for rehydration of the gel pieces, was used. The tryptic cleavage fragments were eluted from the polyacrylamide matrix by shaking with an equal volume of 75% (v/v) trifluoroacetic acid in water for 4h followed by an equal volume of 50% (v/v) trifluoroacetic acid in acetonitrile for an additional 4h. This was repeated twice.

Reversed Phase HPLC of the Cleavage Fragments

The peptides were separated by reversed phase chromatography using a Vydac column (201TP 104, SCI, Science Service). The solvent system used was 0.1% (v/v) trifluoroacetic acid in water (solvent A) and 0.1% (v/v) trifluoroacetic acid in acetonitrile (solvent B). A gradient of 0 to 60% B was performed in 90min using a flow rate of 1ml/min. Fractions were collected manually.

Amino Acid Sequence Analysis

Amino acid sequence analysis of the purified peptides were performed using a gas phase sequencer 470A (Applied Biosystems, Foster City, CA) equipped with a prototype isocratic HPLC system for the identification of the phenylthiohydantoin amino acids [5, 9].

Results and Discussion

The aim of this investigation was to generate peptides suitable for sequence analysis of proteins separated by PAGE since it is usually difficult to digest micro-amounts of proteins after elution from polyacrylamide gels due to large amounts of contaminating detergent, acrylamide oligomers, and salts which strongly inhibit enzymatic cleavage and disturb chromatographic separation. Removal of SDS and salts is tedious and is usually accompanied by severe loss of protein. Two alternative procedures are possible. The enzymatic cleavage can either be performed directly in the gel matrix or after electrophoretical transfer of the protein onto an inert membrane.

The investigations on the nucleoprotein (NP-protein) of various Sendai virus strains may serve as an example. Amino acid exchanges in this protein are thought to play a role in the development of persistent infections. Sendai virus D52 and 6/94 are both lytically infective virus strains, the latter carries a temperature sensitive defect of unknown origin. Sequencing of Sendai 6/94 genomic cDNA clones revealed a frameshift in the carboxy terminal region of the NP gene [10]. In Fig. 2 tryptic peptide maps of electrophoretically isolated nucleoproteins (60 KDa) from different Sendai virus strains are shown. By comparison of the corresponding peptide maps (Fig. 2a and b) a C-terminal peptide could be identified by sequence analysis (unpublished results).

The cell line C1-E8 derived from human brain cells was shown to be persistently infected with Sendai virus [11]. The comparison of peptide maps from the C1-E8 nucleoprotein (Fig. 2c) with the nucleoproteins of the acute infection viruses (D52 and 6/94) (Fig. 2a and 2b) demonstrates the possibility of determining amino acid exchanges.

The second approach (see Fig. 1), the immobilization of the protein by electroblotting and subsequent enzymatic cleavage on the support, has the obvious advantage that all impurities are easily removed during blotting or washing. However, for proteolytic cleavage on the membrane an additional step, the electrophoretic transfer, is necessary which may result in a reduced yield depending on the individual protein [5, 12]. Furthermore, the tight interaction

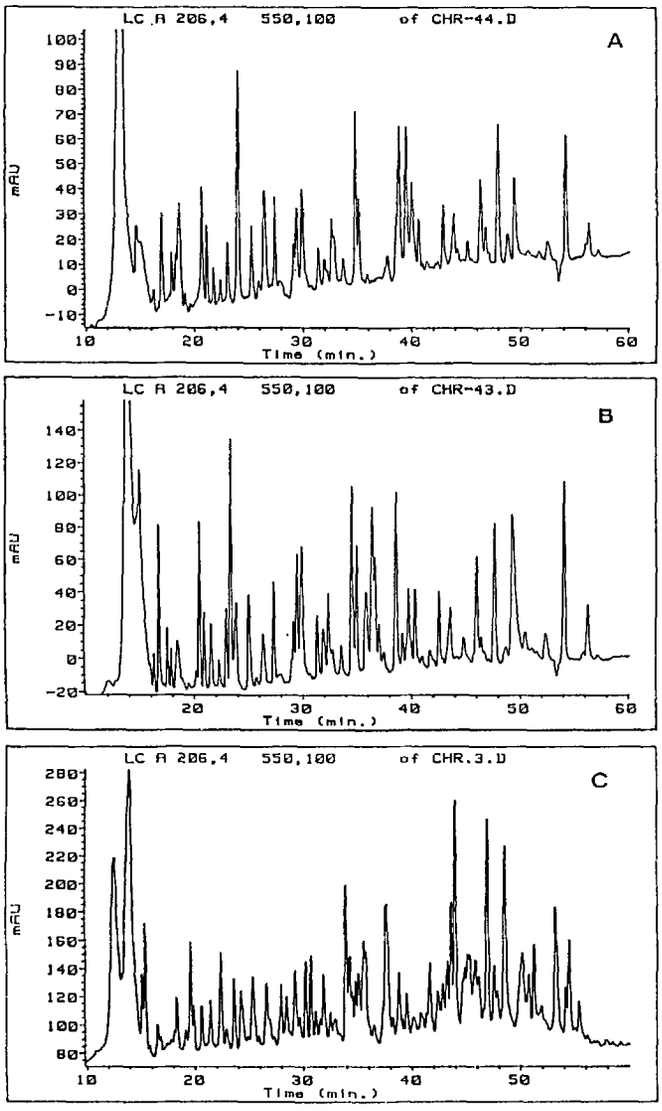

Fig. 2

Tryptic peptide mapping of the nucleoproteins from wildtype Sendai virus D52 (A), from the temperature sensitive mutant Sendai virus 6/94 (B), and from Sendai virus of the persistently infected cell line C1-E8 (C).

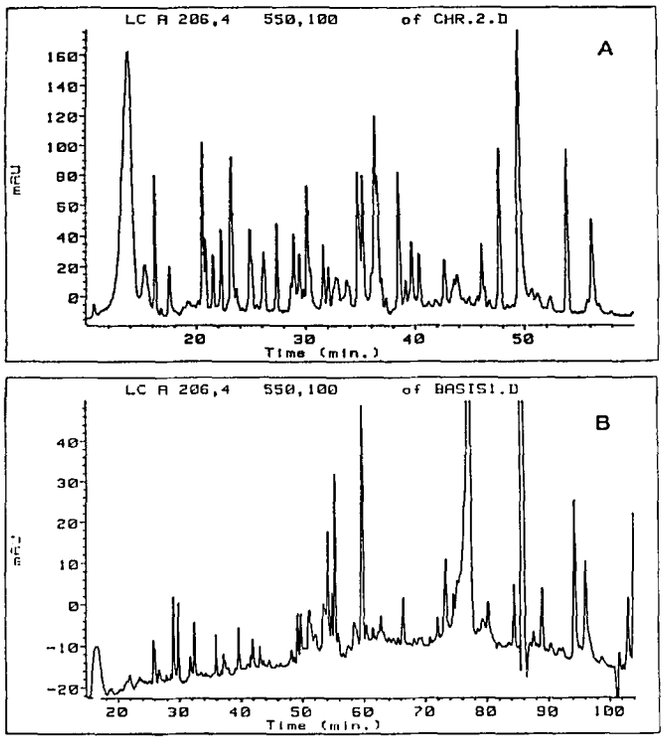

Fig. 3

Tryptic peptide mapping of the nucleoprotein from Sendai virus 6/94. Equal amounts of protein was digested either directly in the polyacrylamide matrix (A), or on silíconized glass fiber sheets after electroblotting.

of both protein and protease with the membrane reduces the accessibility of the protein for the proteinase. This may cause problems in cleavage kinetics and thus prevent complete cleavage. To prevent absorption of proteinase on the membrane, a pretreatment of the membrane is essential as described for nitrocellulose [13].

Equal amounts of complete Sendai virus (strain 6/94) were separated by 10% PAGE, and the NP protein (approximately 300 pmol) was digested either directly in the polyacrylamide matrix or on siliconized glass fiber (SGF) after protein immobilization via electrotransfer. The resulting peptides were eluted as described in the Methods section and separated by reversed phase HPLC (Fig. 3).

From the comparison of the recoveries of identical peptides by both procedures it is obvious that the cleavage in the gel and the subsequent elution from the gel matrix is a more efficient method than enzymatic cleavage on a membrane. Nevertheless, these results indicate that it is possible to obtain amino acid sequence information of proteins immobilized on SGF. Thus, it should be possible to perform first an N-terminal sequence analysis of a protein immobilized on SGF and in the event of N-terminal blockage, to additionally obtain internal sequences by the method currently described. However, the procedure for the recovery of peptides from the siliconized glass fibers has to be improved and is under investigation.

References

[1] *R. C. Allen, C. A. Saravis, H. G. Maurer*, Gel Electrophoresis and Isoelectric Focusing of Proteins, De Gruyter, Berlin 1984.

[2] *R. Aebersold, D. Teplow, L. E. Hood, S. B. H. Kent*, J. Biol. Chem. **261**, 4229–4238 (1986).

[3] *J. Vandekerckhove, G. Bauw, M. Puype, J. Van Damme, M. van Montagu*, Eur. J. Biochem. **152**, 9–19 (1985).

[4] *P. Matsudaira*, J. Biol. Chem. **262**, 10035–10038 (1987).

[5] *C. Eckerskorn, W. Mewes, H. Goretzki, F. Lottspeich*, Eur. J. Biochem. **176**, 509–519 (1988).

[6] NBFR-PIR Protein Sequence Database, Release 16, 1988, Document PRTAPE-0388.

[7] *U. K. Laemmli*, Nature **227**, 680–685 (1970).

[8] *P. H. O'Farrell*, J. Biol. Chem. **250**, 4007–4021 (1975).

[9] *F. Lottspeich*, J. Chromatogr. **326**, 321–327 (1985).

[10] *H. Homann*, Thesis, Universität München (1988).

[11] *W. J. Neubert, P. H. Hofschneider*, Virology **125**, 445–449 (1983).

[12] *C. Eckerskorn, P. Jungblut, J. Klose, F. Lottspeich*, Electrophoresis **9**, 830–838 (1988).

[13] *R. Aebersold, J. Leavitt, L. E. Hood, S. B. H. Kent*, Proc Natl. Acad. Sci. USA **84**, 6970–6974 (1987).

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