Communication

Reproducible High Yield Sequencing of Proteins Electrophoretically Separated and Transferred to an Inert Support*

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Malcolm Moos, Jr., Nga Yen Nguyen, and Teh-Yung Liu

From the Division of Biochemistry and Biophysics, Center for Biologies Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892

A method allowing initial sequencing yields of 60–85% to be consistently obtained from samples prepared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretic transfer is described in detail. Conducting electrophoresis at a pH near neutrality is the single most important of the modifications made to earlier procedures, but pre-electrophoresis in the presence of glutathione or sodium thioglycolate and use of Immobion™ polyvinylidene difluoride membranes all contribute to the success of the technique. When tryptophan was the NH₂ terminus of a protein, the phenylthiohydantoin (PTH)-derivative recovered appeared to be an irreversible oxidation product if pre-electrophoresis was not performed. Following pre-electrophoresis, the PTH-derivative recovered co-migrated with that of unmodified tryptophan, and the recovery was higher. Recovery of methionine as its PTH-derivative was not affected by pre-electrophoresis suggesting that thioglycolate in the electrophoresis buffer during sample separation prevented or reversed oxidation of methionine sulfur but did not protect tryptophan.

Isolation of samples that are free of undesired proteins or other contaminants interfering with phenylthiohydantoin (PTH)¹ analysis in a physical form allowing ready introduction into the gas-phase sequencer remains one of the most troublesome steps in studies of protein structure. Reverse-phase high pressure liquid chromatography in both conventional and narrow bore format (1–3) and electrophoresis followed by elution of separated components (4) have represented useful approaches to this problem. However, many peptides of interest are not amenable to these techniques because of poor recovery of sequenceable material or contaminants introduced by the separation procedures. Several investigators have recently described methods for sample preparation based on electrophoretic transfer of proteins separated by SDS-PAGE to a chemically inert support (5–7). This approach has many potential advantages including operational simplicity, low equipment cost, high throughput, high resolution, and low background in PTH analysis. However, these methods have not found widespread acceptance for a number of reasons. Most of these procedures require manual preparation of the transfer membranes, which can be time consuming and often requires corrosive or toxic reagents. In addition, initial yields have been unacceptably low in the hands of most investigators. Because of the potential utility of this approach, a number of experimental variables were investigated in an attempt to overcome these limitations. This report describes the results of these studies and presents an optimized method.

EXPERIMENTAL PROCEDURES

Materials—Acrylamide, N,N' -methylene bisacrylamide, TEMED, and ammonium persulfate were purchased from Bio-Rad, Marine Colloids, Serva, Boehrager Mannheim, and National Diagnostics and were used without further purification. Buffers were from Serva, Sigma, or Research Organics. SDS was from Bio-Rad or Serva and was sometimes recrystallized from ethanol as recommended by Hunkapiller and Hood (4). IsoGel™ agarose was from Marine Colloids, Serva Blue R was from Serva, N-trimethoxysilylpropyl-N',N'-trimethyl ammonium chloride was from Petrarch Systems, 3,3'-dipentoxycarbonyl-2-mercaptoethanol, and 2-mercaptoethanol, and glyceral were all purchased from Bio-Rad, Marine Colloids, Serva, Boehrager Mannheim, and National Diagnostics. 2-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TES, 2-(2-hydroxyethyl)ethylammonium chloride; BioTris, 2-(2-hydroxyethyl)aminopropyl-N,N',N'-tetramethylpropan-1,3-diol; PVDF, polyvinylidene difluoride; MZE, multiphasic zone electrophoresis; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis. Isolation of samples that are free of undesired proteins or other contaminants interfering with phenylthiohydantoin (PTH)¹ analysis in a physical form allowing ready introduction into the gas-phase sequencer remains one of the most troublesome steps in studies of protein structure. Reverse-phase high pressure liquid chromatography in both conventional and narrow bore format (1–3) and electrophoresis followed by elution of separated components (4) have represented useful approaches to this problem. However, many peptides of interest are not amenable to these techniques because of poor recovery of sequenceable material or contaminants introduced by the separation procedures. Several investigators have recently described methods for sample preparation based on electrophoretic transfer of proteins separated by SDS-PAGE to a chemically inert support (5–7). This approach has many potential advantages including operational simplicity, low equipment cost, high throughput, high resolution, and low background in PTH analysis. However, these methods have not found widespread acceptance for a number of reasons. Most of these procedures require manual preparation of the transfer membranes, which can be time consuming and often requires corrosive or toxic reagents. In addition, initial yields have been unacceptably low in the hands of most investigators. Because of the potential utility of this approach, a number of experimental variables were investigated in an attempt to overcome these limitations. This report describes the results of these studies and presents an optimized method.

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**Electrophoretic Transfer**—Transfer buffer consisted of either 25 mM Tris base, 10 mM glycine, and 0.5 mM dithiothreitol (6) or 10 mM CAPS at pH 9–11, 0.5 mM dithiothreitol (6) and was chilled before use. Transfer was conducted for 10–90 min at either 50 or 100 V, depending on the protein and gel percentage in a particular experiment. Staining of both glass and PVDF membranes was exactly as described (5, 6) except that the glass sheets were found to yield reproducibly lower backgrounds if they were dried by vacuum desication for 30 min prior to staining. Stained bands were excised with a razor blade and each sequenced immediately or stored at −20 °C in sealed Eppendorf tubes.

**Sequencing of Immobilized Proteins**—The excised bands were arranged in a single layer in the upper cartridge block of an Applied Biosystems model 470A gas-phase sequenator and held in place with a trifluoroacetic acid-etched glass fiber filter, which was tamped in place in the usual way. Sequencing and analysis of PTH-derivatives were performed by standard procedures (15). Initial coupling yields were determined from recovery of the NH₂-terminal residue and are presented with respect to the amount of protein loaded onto the gels; the figures given thus reflect losses incurred at any point during sample preparation, electrophoresis, transfer, staining, and sequencing. Repetitive yields were calculated from the recoveries of leucine, isoleucine, valine, alanine, and glutamic acid present in the samples studied.

**RESULTS**

Samples prepared using the Aebbersold technique without modifications (6) gave initial yields ranging from 1 to 12% (Table II). If the separating gel was first subjected to pre-electrophoresis, the initial yield was improved at least 3-fold. When Immobilon™ polyvinyliden difluoride membranes were compared with activated glass sheets in the same experiment, the initial yield was approximately doubled (Fig. 1); repetitive yields were comparable. The procedure using PVDF membranes was more rapid and convenient, and the sharpness of the stained image obtained allowed much more facile separation of closely spaced bands.

Substitution of Jovin system 3328.IV for the Laemmli electrophoresis buffers afforded initial yields of 64–70%, even without pre-electrophoresis (Table II). Similar results were obtained with the neutral phosphate buffer system (11) not shown). With pre-electrophoresis, this figure was further improved to 76–86%. If pre-electrophoresis was carried out for more than 2–4 h, loss of resolution was observed (not shown).

![Fig. 1. Comparison of Immobilon™ PVDF membranes with quaternary ammonium glass.](image)

**TABLE I**

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Upper buffer</th>
<th>1 × Lower buffer</th>
<th>4 × Gel buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>TES (g)</td>
<td>10.07</td>
<td>50.00</td>
<td>21.60</td>
</tr>
<tr>
<td>1 M HCl (ml)</td>
<td>23.66</td>
<td>13.10</td>
<td>10.51</td>
</tr>
<tr>
<td>Final volume (ml)</td>
<td>1000</td>
<td>1000</td>
<td>100</td>
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<tr>
<td>pH (25 °C)</td>
<td>7.26</td>
<td>5.90</td>
<td>6.61</td>
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</table>

*Contains 0.1% SDS.

**TABLE II**

<table>
<thead>
<tr>
<th>Effect of electrophoresis conditions on initial sequencing yield</th>
<th>Protein</th>
<th>Load</th>
<th>Electrophoresis conditions</th>
<th>Initial yield*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>pmol</td>
<td></td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td>100–500</td>
<td>Lea (1–12)</td>
<td></td>
<td></td>
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<tr>
<td>β-Lactoglobulin</td>
<td>100–500</td>
<td>Lea (37–48)</td>
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<tr>
<td>β-Lactoglobulin</td>
<td>100–250</td>
<td>System 3328.IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td>100–250</td>
<td>System 3328.IV with pre-electrophoresis*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Limulus CRP</td>
<td>100</td>
<td>Lea (1–5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Limulus CRP</td>
<td>100</td>
<td>System 3328.IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Limulus CRP</td>
<td>100</td>
<td>System 3328.IV with pre-electrophoresis*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit CRP</td>
<td>100</td>
<td>Lea (5–6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit CRP</td>
<td>100</td>
<td>Lea (5–43)</td>
<td></td>
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</tr>
<tr>
<td>Rabbit CRP</td>
<td>100</td>
<td>System 3328.IV</td>
<td></td>
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<tr>
<td>Sonnatotropin</td>
<td>100</td>
<td>System 3328.IV</td>
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<tr>
<td>Sonnatotropin</td>
<td>100</td>
<td>System 3328.IV with pre-electrophoresis*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Initial yields were determined for the NH₂-terminal residue. The values shown are presented with respect to the amount of protein loaded onto the gels and represent ranges obtained for each condition described in the table over at least five separate experiments.

5–14 h.
2–4 h.
*CRP, C-reactive protein.

**TABLE III**

<table>
<thead>
<tr>
<th>Tryptophan initial yield</th>
<th>Samples of Limulus 18,000 protein were treated as described and blotted in parallel or spotted directly onto precycled Polybrene and inserted directly into the sequenator. The retention time of PTH-tryptophan was 18.9 min; that for PTH-tryptophan was 18.25 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrophoresis conditions</td>
<td>Amino acid recovered</td>
</tr>
<tr>
<td></td>
<td>Trp-1</td>
</tr>
<tr>
<td>None (direct application to sequenator)</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>System 3328.IV</td>
<td>Kynurenine</td>
</tr>
<tr>
<td>System 3328.IV with pre-electrophoresis</td>
<td>Tryptophan</td>
</tr>
</tbody>
</table>

Further recrystallization of the commercial SDS used during electrophoresis did not affect initial yields or the appearance of artifact and background peaks (not shown). Similarly, the source of acrylamide did not appear to influence results substantially. Over the range tested (100–500 pmol), the percent initial yield was independent of sample load.

The effects of various sample treatments on tryptophan yield are presented in Table III. When the Limulus 18,000 protein was applied directly to the sequenator, the yield for the NH₂-terminal tryptophan was 78%. SDS-PAGE and blotting decreased the yield to 20–26% if pre-electrophoresis was omitted. In this instance, the PTH-derivative obtained from the first cycle of Edman degradation comigrated with authen-
tic PTH-kynurenine, an oxidation product of tryptophan. Following pre-electrophoresis, the PTH product migrated in the position expected for PTH-tryptophan, and the yield in the first cycle was 25–28%. There was no indication of progressive loss with heating under the conditions tested. The yield of NH₂-terminal methionine was comparable whether the sample was spotted directly onto preycycled Polybrene or subjected to electrophoresis and blotting with or without pre-electrophoresis.

**DISCUSSION**

This report presents a convenient method which has allowed operators in several different laboratories to obtain consistently high initial sequencing yields with a variety of proteins. Each modification described was tested using identical aliquots of standardized proteins to facilitate comparisons between laboratories and to eliminate variation due to the nature of the sample or its preparation. All initial yield figures were based on amounts of sample applied to the gel to eliminate confusion regarding sources of sample loss and to provide a realistic estimate of the overall efficiency of the manipulations evaluated.

Unacceptably low initial sequencing yields have been the most consistently observed and troublesome of the problems encountered with SDS-PAGE/electrophoretic transfer procedures. Several pieces of evidence suggested that the most likely cause for this difficulty was NH₂-terminal blockage occurring during electrophoresis. When β-lactoglobulin or sperm whale myoglobin were spotted directly onto activated glass, soaked in transfer buffer, and stained, excellent initial sequencing yields were obtained.³

In addition, several anecdotal reports suggested that different lots of gel polymerization reagents may influence the quality of results obtained. Finally, in one experiment,⁴ a protein which had been subjected to SDS-PAGE and transferred to activated glass and initially failed to yield sequence information was then exposed to cyanogen bromide and returned to the sequenator. The expected sequence from the COOH-terminal CNBr fragment was obtained in good yield.

These observations suggested that strategies to eliminate polymerization by-products remaining in the gel would be of benefit. The first of these to be tried was pre-electrophoresis of the separating gel in separating gel buffer containing reduced glutathione or subsequently, thioglycolate. It was hoped that this would have three effects: 1) removal of charged impurities; 2) reduction of peroxides and residual radicals; and 3) scavenging of uncharged reactive species such as acrylamide monomer, free or incorporated acrolein, or other reactive carbonyl compounds. Since the standard Laemmli system uses a different buffer in the separating and stacking gels, only the separating gel could be treated in this way; stacking gels were, therefore, prepared with agarose.

Even using Immobilon™ membranes and pre-electrophoresis, initial sequencing yields were seldom above 50%. Further, initial yield was independent of sample load over the range examined. If a limited quantity of highly reactive precycled Polybrene-coated glass fiber discs (Table II), and contaminant and artifact peaks were much lower (not shown). This is especially important for diphenylurea, which comigrates with PTH-tryptophan. Since these yields are presented with respect to amounts of sample loaded onto the gel, all sources of loss are taken into account. In all cases, repetitive yields have been 94–96%, even though Polybrene was not used in any of our experiments. Indeed, omission of Polybrene likely contributes to reduction of undesired peaks. We, therefore, feel that the procedure incorporating the use of PVDF membranes and an electrophoresis buffer system operating close to neutral pH should allow the technique to be useful on a routine basis.

System 3328.IV is no more difficult to use than the Laemmli system, involving the same number of stock solutions. We recommend pre-electrophoresis for 30 min to assure recovery of PTH-tryptophan and to improve the initial yields of other amino acids. It should be noted that if pre-electrophoresis is carried out for more than 2–4 h at 8 mA/minigel in system 3328.IV, resolution is impaired, presumably due to the accumulation of electrolysis products. Gels may be stored overnight after pre-electrophoresis in the glutathion-containing buffer with no ill effects, however.

A few additional practical considerations deserve special comment. Different sources of acrylamide gave similar results (not shown), despite unpublished reports to the contrary. This does not exclude the possibility that certain batches of acrylamide, perhaps containing relatively high concentrations of acrylic acid, acrolein, or other reactive contaminants, some of which may be generated on storage, may in many cases impair results. However, it would seem from our data that these problems are of secondary importance when the operating pH of the separation is decreased. Some samples appear to run as aggregates if heated at 55 °C as recommended by Hunkapiller and Hood (4). In our experience, tryptophan yields do not appear to be adversely affected by more extensive heating.

Use of a piperasinesulfonic acid buffer (6) avoids the problem of a PTH-glycine signal in early cycles (5). Though CAPS at relatively high pH has worked well in our hands, it would be

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² M. Moos and K. Seamon, unpublished observations.
³ M. Hunkapiller, personal communications.
reasonable to use a similar buffer with a lower pK (e.g. Hepes) for proteins requiring prolonged transfer to minimize pH-dependent NH\textsubscript{2} terminus blockage at this step. The pH chosen should be above the isoelectric point of the desired protein.

The 18,000 protein used in these studies has tryptophan at the NH\textsubscript{2} terminus.\textsuperscript{5} The initial yields obtained when this peptide was subjected to SDS-PAGE and blotting were consistently lower than for C-reactive protein and \(\beta\)-lactoglobulin. Further, if pre-electrophoresis was not performed, the PTH-derivative recovered appeared to be an irreversible oxidation product of tryptophan. This suggests that oxidizing equivalents remained in the gel matrix. Following pre-electrophoresis, the PTH derivative recovered comigrated with unmodified tryptophan and the yield was higher. When methionine was at the NH\textsubscript{2} terminus, there was no apparent decrement in initial yield, Thus, it would appear that the mercaptoacetic acid present in the electrophoresis buffer prevented or reversed oxidation of methionine but was not able to protect tryptophan.

In summary, we conclude that difficulty in utilizing protein transfer procedures for microsequencing may stem from at least three sources: poor binding to the inert support, NH\textsubscript{2}-terminal blockage during electrophoresis, and oxidation of susceptible amino acids. Immobilon\textsuperscript{TM} polyvinylidene difluoride membranes conveniently address the first of these problems. Since very low initial yields were often obtained even with the PVDF membranes when using the Laemmli system (Table II), our results suggest that NH\textsubscript{2}-terminal blockage and/or chemical degradation of labile residues are more serious considerations. Whether caused by poorly characterized oxidizing species remaining after polymerization (19), incorporated or unincorporated acrolein, or acrylamide monomer (19, 20), most of these difficulties may be resolved by performing electrophoresis near neutral pH to reduce the reactivity of NH\textsubscript{2} termini and by pre-electrophoresis to remove or inactivate undesired compounds remaining in the gel. The result of these modifications is a reliable system for sample preparation that should be of particular utility for sequencing of large insoluble peptides (e.g. those derived from receptors and other hydrophobic insoluble membrane proteins) difficult to isolate by other means.

Acknowledgements—We wish to thank Drs. K. Seamon, N. Gold- man, L. Callahan, M. Huntkiller, A. Chrambach, and R. Aebersold for many helpful discussions during the course of this study. The technical assistance of John Ewe11 is also gratefully acknowledged.

REFERENCES

\textsuperscript{5} N. Fujii, N. Nguyen, and T-Y. Liu, unpublished observations.