Biol. Chem. Hoppe-Seyler
Vol. 371, pp. 675–685, August 1990

A Facile Method for the Isolation and Preparation of Proteins and Peptides for Sequence Analysis in the Picomolar Range

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(Received 22 December 1989/8 May 1990)

Summary: We report a new and facile extraction method of proteins and polypeptides in the range of 100 to 1 kDa previously separated by high-resolution SDS/polyacrylamide-gel electrophoresis. Proteins and polypeptides obtained by chemical or proteolytic cleavage of proteins can directly be applied to high-sensitivity N-terminal amino-acid sequence analysis by gas-phase sequencing. The Coomassie Blue-stained protein bands are eluted from the gel slices with 0.1M sodium acetate buffer, pH 8.5, 0.1% SDS in high yield and directly applied to the filter disc of the gas-phase sequencer.

The superior efficiency for the isolation of proteins and polypeptides from polyacrylamide gels for micro-sequencing has been documented by a quantitative comparison of the procedure described here and the favoured electroblot-transfer method using ^{14}C-labelled marker proteins.

This highly efficient isolation has been successfully reproduced and applied to the analysis of a variety of proteins and peptides with rather divergent physical properties, particularly to hydrophobic peptides isolated from SDS/polyacrylamide gels. The electrophoretic transfer onto activated glass filters, Immobilon membranes (polyvinylidene-difluoride membranes), siliconized or chemically activated glass fiber supports can be omitted. The method considerably simplifies and speeds up the isolation, and improves the sensitivity as compared to the electroblotting procedures due to the reproducibly high recoveries.

Eine einfache Isolierungs- und Präparationsmethode für die Sequenzanalyse von Proteinen und Polypeptiden im Picomol-Bereich

Zusammenfassung: Wir berichten über eine neue und vereinfachte Methode für die Isolierung von Proteinen oder Polypeptiden in der Größenordnung von 100 bis 1 kDa, die vorher durch hochauflösende SDS/Polyacrylamid-Gelelektrophorese getrennt wurden. Die aus chemischen und proteolytischen Spaltungsreaktionen resultierenden Peptide sind in einer für die hochempfindliche N-terminale Aminosäuresequenz-Analyse direkt geeigneten Form. Die Coomassie-Blue-gefärbten Proteinbanden werden aus den Gelstückchen mit 0.1M Natriumacetatpuffer, pH 8.5, 0.1% SDS in hoher Ausbeute eluiert und direkt auf die Filterscheibe des Gasphasensequenators übertragen.

Ein quantitativer Vergleich zwischen der hier beschriebenen Elutionsmethode und der meist gebräuchlichen Elektroblot-Transfer-Methode für die Isolierung von Proteinen und Peptiden aus Polyacrylamidgelen für die Mikrosequenzierung wurde mit ^{14}C-radioaktiv markierten Marker-Proteinen durchgeführt und dokumentiert die überlegene Wirksamkeit der Puffer-Elution.

Enzyme:
Trypsin (EC 3.4.21.4).

Abbreviations:
HPLC, High-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PLP, proteolipid protein (of central nervous system myelin); PTH, phenylthiohydantoin derivative; SDS, sodium dodecyl sulfate.

Diese Methode wurde für die Analyse von Proteinen und Polypeptiden mit sehr unterschiedlichen physikalischen Eigenschaften, besonders von hydrophoben Polypeptiden, erfolgreich und reproduzierbar angewandt. Die elektrophoretische Übertragung auf aktivierte Glasfaserfilter, Immobilon-Membran (Polyvinylidendifluorid-Membranen) und silanisierte Glasfaserfilter werden überflüssig. Die Methode vereinfacht und beschleunigt die Isolierung und verbessert die Empfindlichkeit entscheidend im Vergleich mit der Elektroblot-Methode durch die reproduzierbar hohe Ausbeute.

Key words: Protein isolation, peptide isolation, sequence analysis in picomolar range.

The isolation of proteins and polypeptides in sufficient purity for amino-acid sequence analysis in the picomolar range is still a challenging problem. The main difficulty encountered is the recovery of a respective polypeptide entrapped in the polyacrylamide-gel matrix after the separation by SDS gel electrophoresis of complex mixtures. Because of its convenience and high resolution SDS/PAGE has increasingly become the method of choice for the final isolation and purification step. This is of particular relevance for low abundance proteins of different origin such as DNA binding proteins, growth factors, receptors etc. Their complete structure is mainly derived from cDNA nucleotide sequence data. The key to these are frequently partial amino-acid sequence data for the synthesis of the corresponding oligonucleotides as probes for the screening procedures.

A commonly used isolation procedure of submicro, subnano and picomole amounts is the electroelution of the stained protein bands from the gel slices[1], also the HPLC separation with volatile solvents by gradient elution[2] and the direct elution of proteins with guanidinium thiocyanate which was recently published[3]. All techniques have numerous drawbacks such as variable recovery, they are time-consuming and require expensive equipment. The procedure described by Prussak et al.[3] who stained their protein lanes in the polyacrylamide gel with KCl is limited to 3–5 μg of each protein band and involves some precipitation steps after protein elution to allow the following proteolytic treatment.

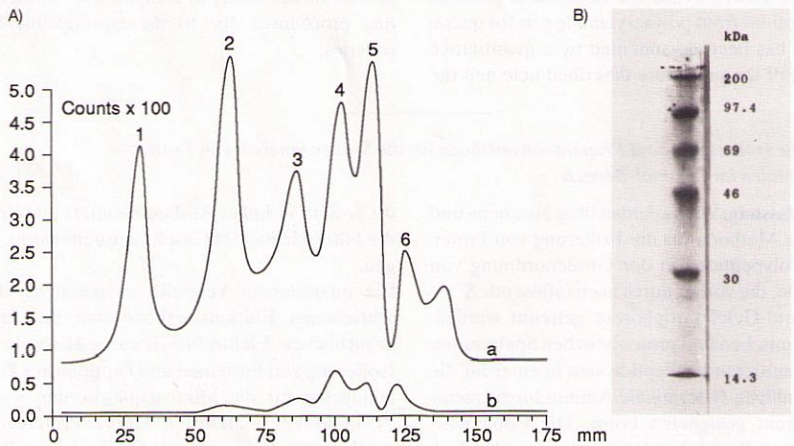


Fig. 1. Radioscans of ^{14}C-labelled marker proteins separated by SDS/PAGE. Gels were dried on Whatman paper and scanned with a thin-layer radiochromatogram scanner Rita 3200, Raytest (A), or autoradiographed using a Kodak XAR-5 film (B).

a) Scan before and b) after elution with sodium acetate/SDS buffer, ^{14}C-Marker proteins: 1, lysozyme 14.3 kDa; 2, carbonate dehydratase 30 kDa; 3, ovalbumin 46 kDa; 4, bovine serum albumin 69 kDa; 5, phosphorylase *b* 97.4 kDa; 6, myosin 200 kDa; band 7 corresponds to aggregated protein at the origin. Comparison of the two radioscans a and b shows an elution yield of 100% for peak 1; 95% elution for peak 2; 95% for peak 3; 87% for peak 4; 92% for peak 5; 79% for peak 6.

b) residual radioactive marker proteins of a polyacrylamide gel strip loaded with the same aliquot of radioactive markers for PAGE as in a) after buffer elution.

Currently the sensitivity of detection of PTH amino-acid derivatives, which we detect in the range of 5 pmol with the equipment and the conditions described below, and the isolation and preparation of protein bands separated by SDS/PAGE are the limiting factors.

The direct electrophoretic transfer of polypeptides from SDS/polyacrylamide gels to several modified glass fiber supports has been introduced recently. Glass fiber filters have been silanized either with *N*-trimethoxysilylpropyl-*N*,*N*,*N*-trimethylammonium chloride or aminopropyltrioxysilane[4] or with poly(methyl-3,3,3-trifluoropropylsilane)[5]. Proteins were also electroblotted onto polyvinylidene difluoride membranes[6] or polybrene pretreated glass fiber discs[7] and Immobilon membranes[8]. Finally the electrotransfer and covalent immobilization of proteins can be carried out to 1,4-phenylene-diisothiocyanate linked to aminopropyl-glass filters for high sensitivity sequence analysis[9].

We report here a simple and reproducible method for the highly efficient recovery of proteins and polypeptides from SDS/PAGE ranging in molecular masses from 1 to 100 kDa by elution in a suitable buffer system. The elution mixture can be directly applied to the filter disc of the gas-phase sequencer for N-terminal and internal sequence analysis. Our procedure has been compared with electroblotting procedures (Immobilon and silanized glass-fiber filters) with proteolytic fragments of three proteins, myoglobin, lysosomal sphingomyelinase and proteolipid protein from bovine brain, respectively, all very different in hydrophobicity.

Results

In the course of our protein analytical work preceding and accompanying cloning and gene expression studies we met the need for a fast and highly efficient separation, isolation and preparation procedure of

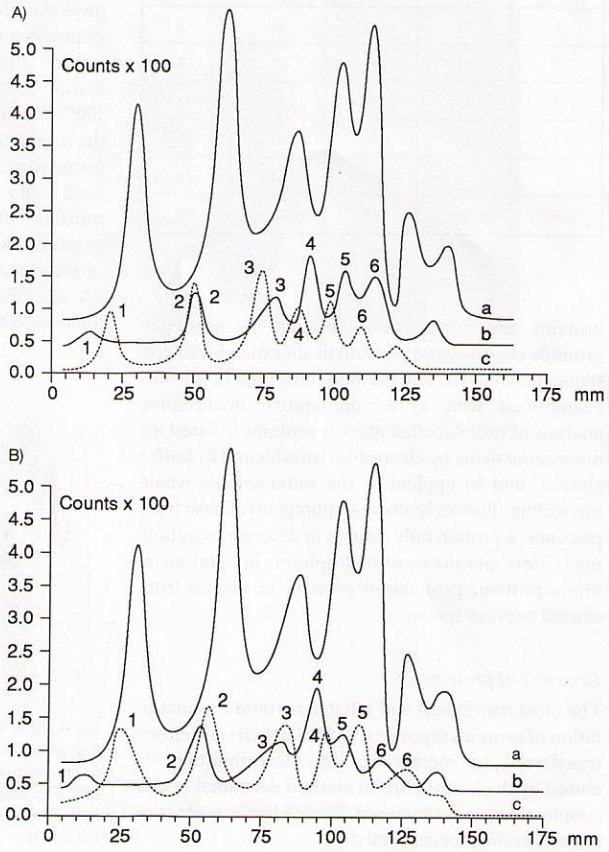


Fig. 2. Radioscans of activated glass fiber membranes (Biometra) to which the ^{14}C-labelled marker proteins (as in Fig. 1) have been transferred by electroblotting for A: 2 h and B: 12 h. Radioscan a: radioactivity of the marker proteins in the gel before electroblotting; b: radioactivity of the marker proteins after electroblotting onto the glass fiber membrane; c: remaining radioactivity of the marker proteins in the gel after electroblotting.

The sensitivity of the scanner was the same for the gel before protein transfer and for the membrane. The blotting buffer contained 20% methanol as described by Eckerskorn et al.[5].

Fig. 3. Electroblots of marker proteins.

A) Prestained standard proteins (BRL) with standard transfer buffers (20% methanol, 50mM boric acid, pH 9.0).
Lanes: 1, Protein markers; 2, protein remaining in 12% acrylamide gel after 2 h; 3, after 12 h blotting time; 4 and 5, marker proteins transferred onto glass fiber membrane (Biometra) after 2 h (lane 4) and 12 h (lane 5).

B) Same as A except for the transfer buffer (10% methanol, 50mM boric acid, pH 9.0, 0.1% SDS).
Lanes: 1, Prestained protein standard (BRL); 2, protein remaining in the gel after 2 h; 3, after 12 h blotting time; 4, glass fiber membrane, blotting time: 2 h; 5, blotting time: 12 h; 6 and 7 prestained marker proteins collected on a second membrane placed behind the primary filter, blotting time 12 h.

C) Same as B except for ^{14}C-labelled marker proteins instead of prestained proteins. Lane a is identical to lane a in Fig. 1. Lanes b and c compare the radioactivities captured on the glass fiber membrane (b) and the residual activity in the gel (c).
Protein recovery was quantified by radioscanning with the following distribution: Recording b) 1, 8%; 2, 15%; 3, 31%; 4, 36%; 5, 19%; 6, 17%; c) 1, 2%; 2, 5%; 3, 6%, 4, 6%; 5, 12%; 6, 7%.

Table. Evaluation of scans b (protein recovery on glass fiber membrane) and c (protein remainder in the gel after electroblotting) in Fig. 2A and B.

Duration of electroblot	A) 2 h		B) 12 h	
Peak no.	b) Recovery [%]	c) Remainder [%]	b) Recovery [%]	c) Remainder [%]
1	38	7	45	5
2	33	12	38	7
3	29	32	33	24
4	30	36	43	18
5	26	27	34	21
6	18	45	21	37

proteins and polypeptides obtained by cyanogen bromide cleavage and proteolytic digestion for microsequence analysis. The method described below was examplified with a) a comparative quantitative analysis of radiolabelled marker proteins isolated for microsequencing by electroblot transfer and by buffer elution, and b) applied to the water-soluble whale myoglobin, human lysosomal sphingomyelinase from placenta, a protein only soluble in detergent-containing buffers and the most hydrophobic integral membrane protein, proteolipid protein, of myelin from central nervous tissue.

Recovery of proteins

The most convenient and reliable method for quantitation of proteins separated by SDS/PAGE and either transferred to membranes by electroblotting or eluted by the buffer elution method described in this communication is the use of ^{14}C-labelled marker proteins of known specific activity.

We used a set of six marker proteins. Their approximate molecular masses were in the range between 200 and 14.3 kDa. They were separated as described under Methods, identical examples in adjacent lanes of the polyacrylamide gel (Fig. 1). The reference lane was dried on Whatman paper. The second lane was eluted with the sodium acetate – SDS buffer as described under Methods and the residual radioactive proteins also transferred to Whatman paper for radioscanning. Fig. 1 compares the radioscans before (a) and after (b) the elution procedure. Our buffer elution procedure allows a recovery between 80 and 100%. The distribution of the radioactivity is listed in the legend to Fig. 1. If, however, other lanes are transferred to activated glass fiber filters by electroblotting for 2 h and 12 h, respectively, not only the inefficient transfer but also the well-known spreading of the bands of the transferred marker proteins can be seen in the radioscans of the electroblot membrane, Fig. 2A and 2B. We also observed that during the prolonged electroblotting part of the transferred proteins

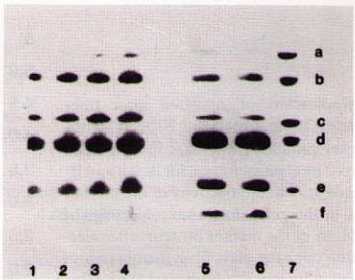
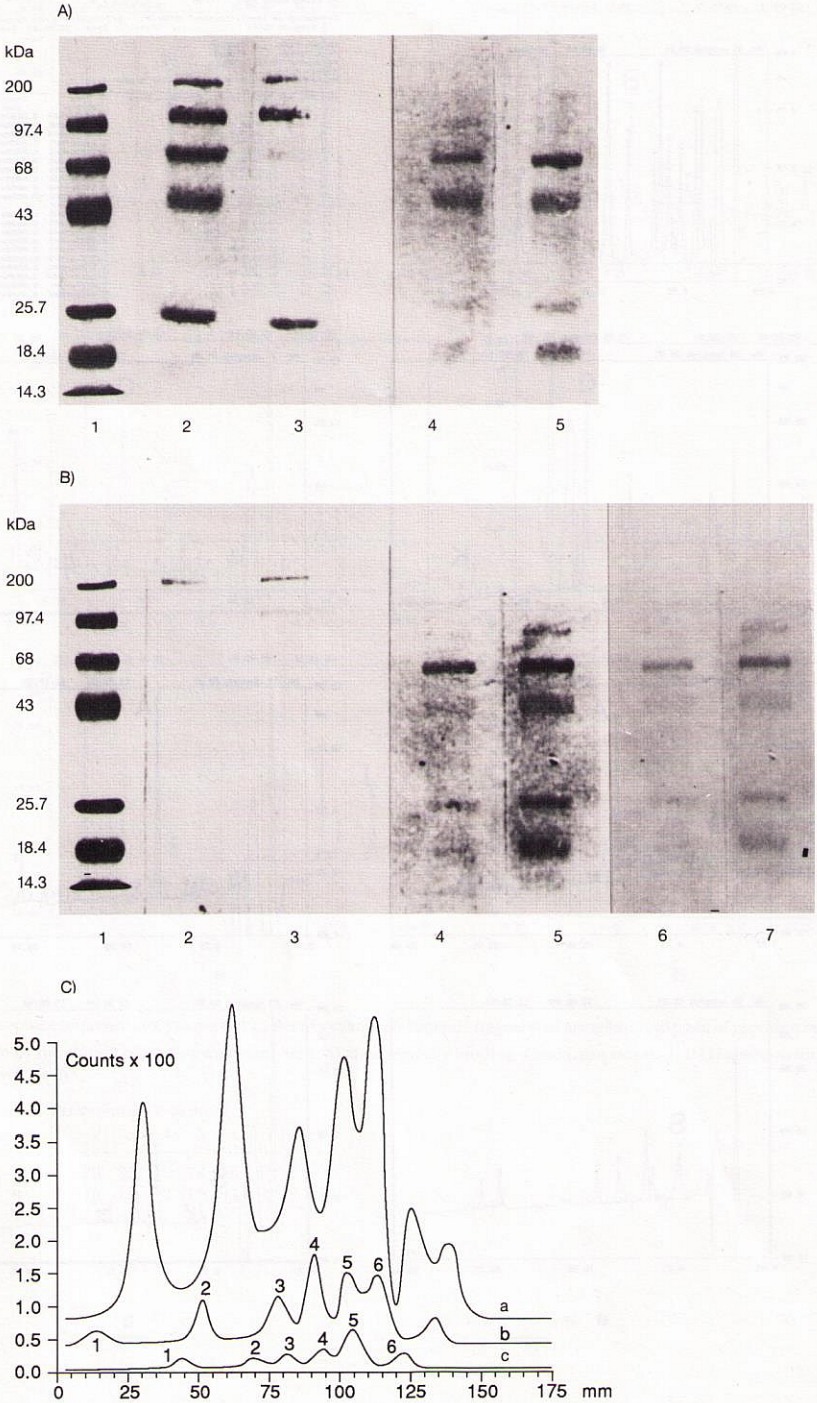


Fig. 4. SDS/PAGE of cyanogen bromide fragments of myoglobin, stained with Coomassie blue. Lanes: 1–4, Semi-dry blot of fragments to derivatized glass fiber membrane. 5, 6, Separation of CNBr fragments of myoglobin on Tricine SDS/PAGE. 7, SDS 17 marker (Sigma): a, 17.0; b, 14.5; c, 10.7; d, 8.2; e, 6.3; f, 2.5 kDa.


A)
kDa
200
97.4
68
43
25.7
18.4
14.3
1
2
3
4
5
B)
kDa
200
97.4
68
43
25.7
18.4
14.3
1
2
3
4
5
6
7
C)
Counts x 100
5.0
4.5
4.0
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3.0
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2.0
1.5
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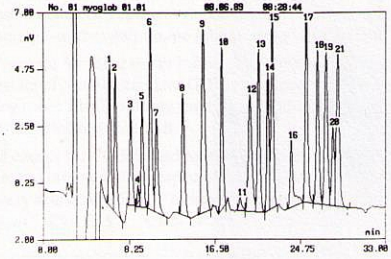
1



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Chromatogram Identification MYOGLOB: BrCN-Spaltung von Myoglobin
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Method File..... MYOGLOB .....
Peak Table..... MYOGLOB .....
Parameter Table. MYOGLOB .....
Result File..... .....
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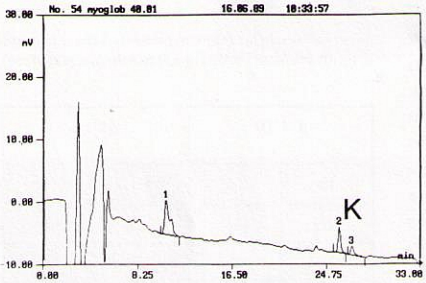
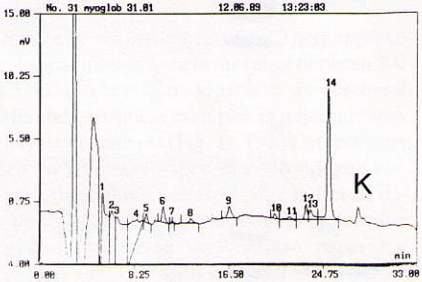
No.	PNo	Ret.Time min	Type	Substance Name	Area mVmin	Amount pMol	Rel.Area %
1	1	6.31	ML	Asp	1.4409e+000	0.0000e+000	4.38
2	2	6.86	M R	Asn	1.5507e+000	0.0000e+000	4.62
3	3	8.35	MLR	Gln	1.0694e+000	0.0000e+000	3.19
4	4	9.04	RuL	Thr	1.1811e-001	0.0000e+000	0.35
5	5	9.46	M r	Gly	1.2445e+000	0.0000e+000	3.72
6	6	10.26	M1	Glu	2.0427e+000	0.0000e+000	6.10
7	7	10.86	M R	DMPTU	1.2770e+000	0.0000e+000	3.81
8	8	13.48	MLR	Ala	1.4988e+000	0.0000e+000	4.45
9	9	15.34	MLR	His	2.9624e+000	0.0000e+000	8.85
10	10	17.16	MLR	Tyr	1.7338e+000	0.0000e+000	5.17
11	7	18.94	ML	?	1.8584e-001	?	0.55
12	11	19.84	M	Arg	1.9585e+000	0.0000e+000	5.85
13	12	20.69	M r	Pro	1.9685e+000	0.0000e+000	5.88
14	13	21.59	M1	Met	1.4282e+000	0.0000e+000	4.26
15	14	22.01	M R	Val	2.2215e+000	0.0000e+000	6.64
16	15	23.03	MLR	DPTU	1.0025e+000	0.0000e+000	2.99
17	16	25.20	M1	Trp	2.2971e+000	0.0000e+000	6.86
18	17	26.35	M	Phe	1.9736e+000	0.0000e+000	5.89
19	18	27.19	M	Ile	2.1813e+000	0.0000e+000	6.51
20	19	27.88	M	Lys	9.7374e-001	0.0000e+000	2.91
21	20	28.35	M R	Leu	2.3670e+000	0.0000e+000	7.07

2





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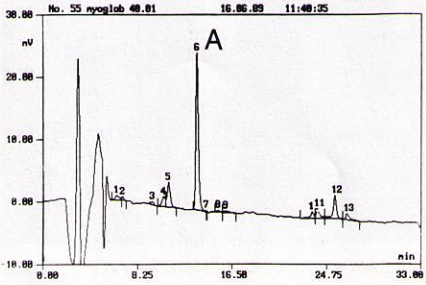

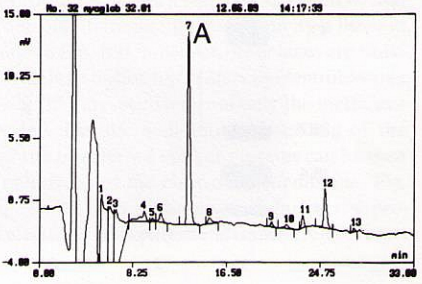

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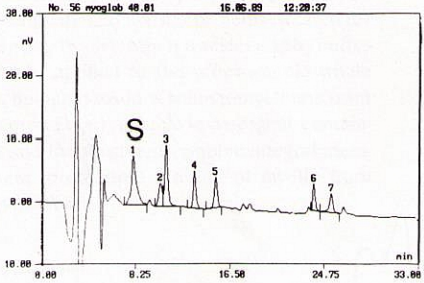

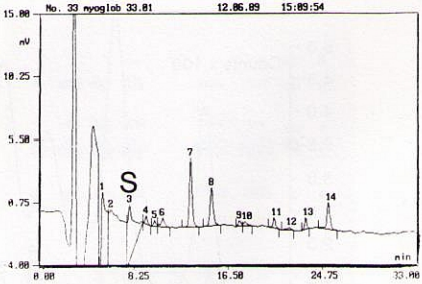

a b

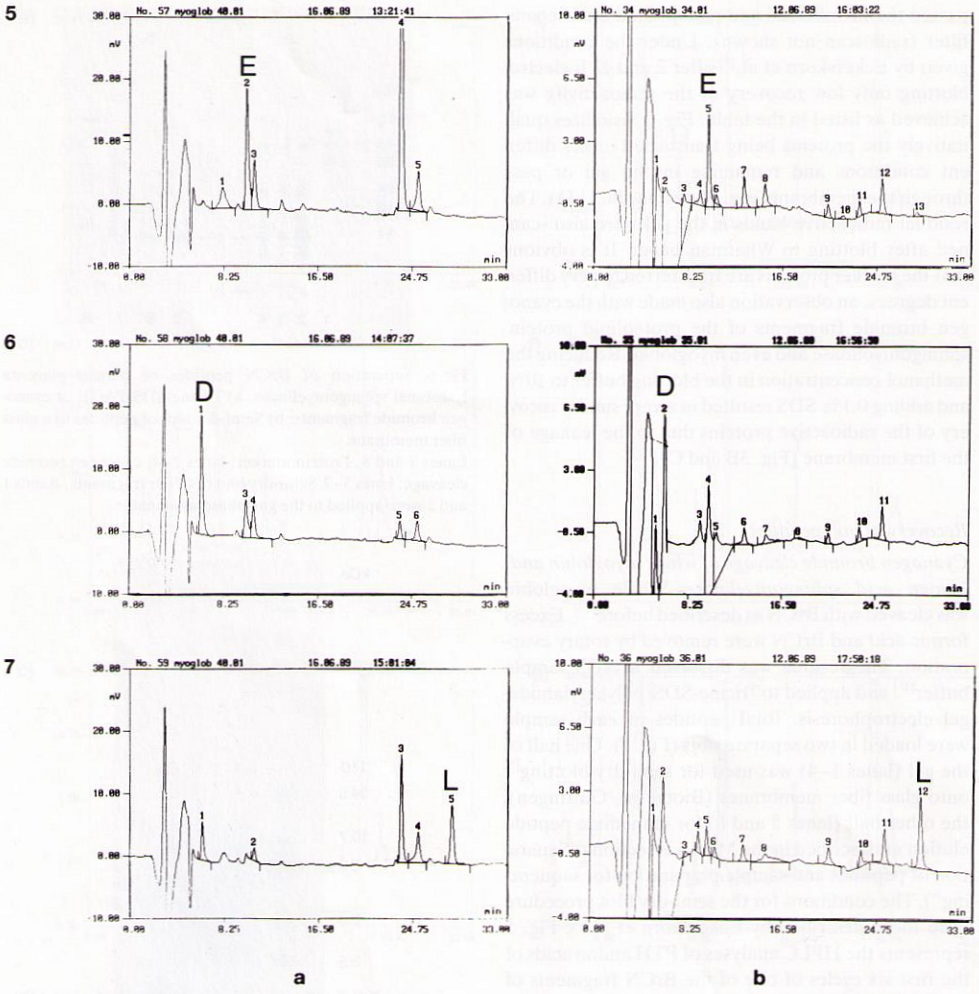

Fig. 5. Gas-phase sequence analysis over six cycles of a cyanogen-bromide fragment of myoglobin (49 pmol of peptide e in Fig. 4). a) Isolation by the elution procedure described here, b) after semi-dry blotting. Chromatogram no 1: PTH amino-acids standard; no. 2–7: cycles 1–6.

Initial yields of PTH amino acids in pmol

Chromatogram no.	2	3	4	5	6	7
a)	20	35	15	28	26	15
b)	16	18	6	18	11	8

passed the membranes and precipitated on a second filter (radioscan not shown). Under the conditions given by Eckerskorn et al.[5] after 2 and 12 h electroblotting only low recovery of the radioactivity was achieved as listed in the table. Fig. 3 visualizes qualitatively the proteins being transferred under different conditions and remaining in the gel or pass through the membrane, e.g. lysozyme, 14.3 kDa. The residual radioactive bands in the gel were also scanned after blotting to Whatman paper. It is obvious that the marker proteins are transferred to very different degrees, an observation also made with the cyanogen bromide fragments of the proteolipid protein, sphingomyelinase and even myoglobin. Reducing the methanol concentration in the blotting buffer to 10% and adding 0.1% SDS resulted in a very similar recovery of the radioactive proteins due to the leakage of the first membrane (Fig. 3B and C).

Recovery of polypeptides

Cyanogen bromide cleavage of whale myoglobin and human acid sphingomyelinase: Whale myoglobin was cleaved with BrCN as described before[10]. Excess formic acid and BrCN were removed by rotary evaporation. The residue was dissolved in SDS sample buffer[11] and applied to Tricine-SDS/polyacrylamide-gel electrophoresis. Total peptides of each sample were loaded in two separate slots (Fig. 4). One half of the gel (lanes 1–4) was used for semi-dry blotting[5] onto glass fiber membranes (Biometra, Göttingen), the other half (lanes 5 and 6) for immediate peptide elution as described in the Methods section ("Separation of peptides and sample preparation for sequencing"). The conditions for the semi-dry blot procedure were those described by Eckerskorn et al.[5]. Fig. 5 represents the HPLC analyses of PTH amino acids of the first six cycles of one of the BrCN fragments of myoglobin isolated by elution (a) and by semi-dry blotting (b). Notice the three times higher sensitivity applied in the detection of the PTH amino acid of the electroblotted sample. The sequence KASEDL corresponds to positions 56–61.

Similarly lysosomal sphingomyelinase purified from human placenta was localized by Coomassie Blue staining of the preparative SDS/polyacrylamide gel. The gel piece with the sphingomyelinase 70-kDa band was excised, washed with water and BrCN-cleaved in the gel slice. The solution was concentrated by rotary evaporation. Peptides were separated by Tricine-SDS/polyacrylamide-gel electrophoresis as demonstrated in Fig. 6. Three of the six peptide lanes (5, 6, 7) were used for semi-dry electroblotting to a siliconized glass fiber membrane. Bands 1 and 2 of lanes 2, 3 and 4 were eluted and sequenced directly.

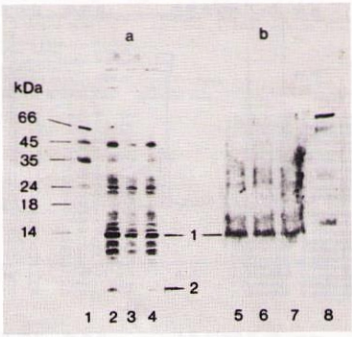

Fig. 6. Separation of BrCN peptides of human placenta lysosomal sphingomyelinase. a) Tricine-SDS/PAGE of cyanogen bromide fragments; b) Semi-dry blot of peptides to a glass fiber membrane.
Lanes 1 and 8, Protein marker; lanes 2–4, cyanogen bromide cleavage; lanes 5–7, Semi-dry blot of CNBr fragments. Bands 1 and 2 were applied to the gas-phase sequenator.

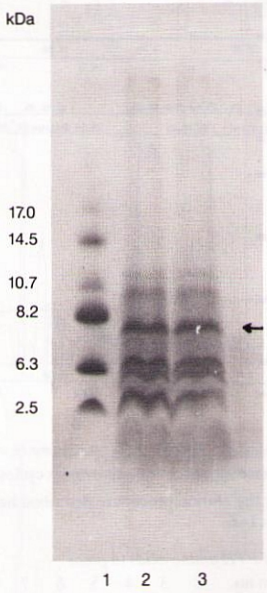

Fig. 7. Tricine SDS/PAGE of tryptic digests of the strongly hydrophobic PLP.
Lane 1, Protein marker (SDS 17 marker, Sigma), lanes 2 and 3, tryptic peptides of PLP.

Tryptic fragments of proteolipid protein isolated from myelin of bovine brain: Myelin of bovine brain was isolated according to Norton[12] and extracted with chloroform-methanol. Proteins of the organic extract

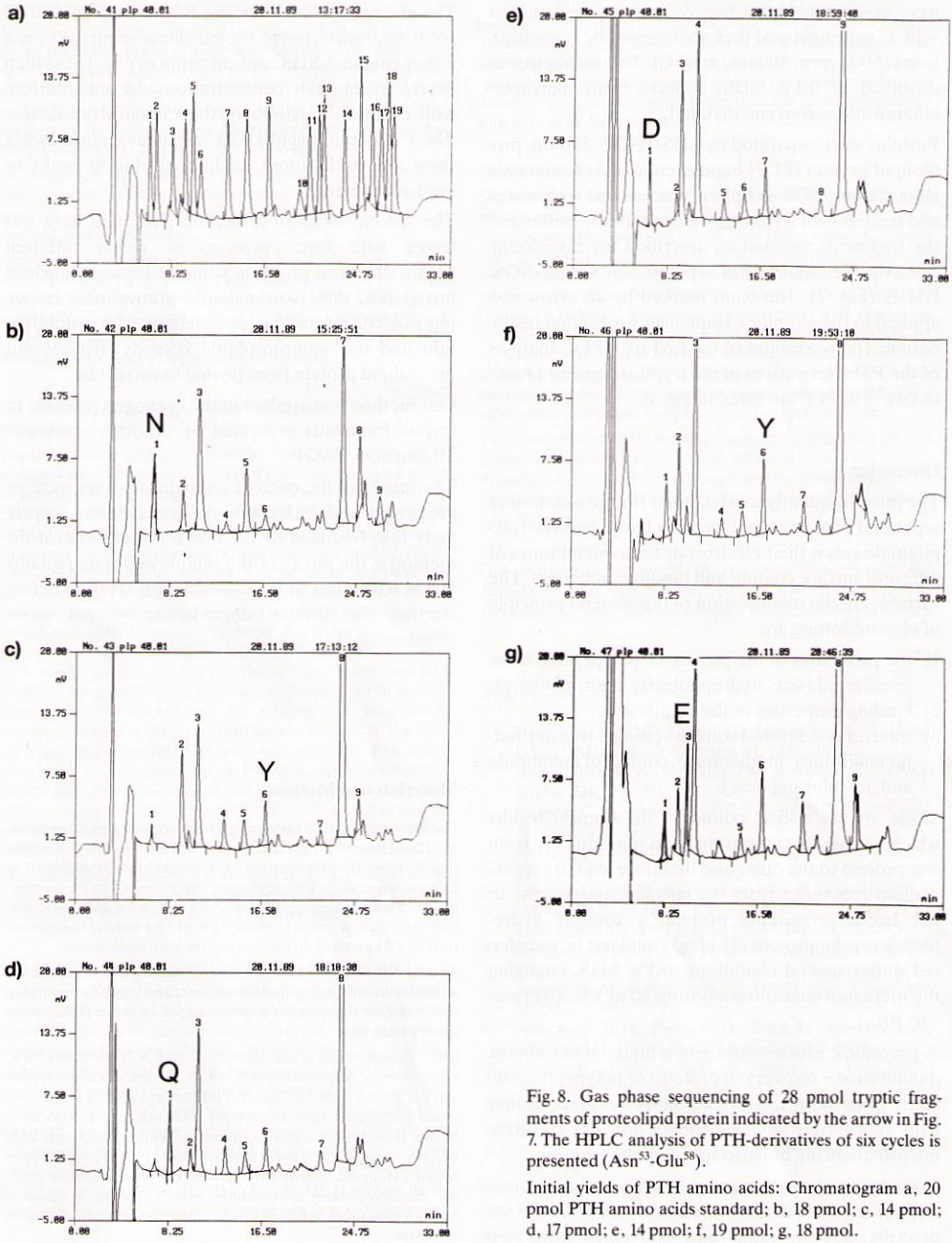

Fig. 8. Gas phase sequencing of 28 pmol tryptic fragments of proteolipid protein indicated by the arrow in Fig. 7. The HPLC analysis of PTH-derivatives of six cycles is presented (Asn^{53}-Glu^{58}).

Initial yields of PTH amino acids: Chromatogram a, 20 pmol PTH amino acids standard; b, 18 pmol; c, 14 pmol; d, 17 pmol; e, 14 pmol; f, 19 pmol; g, 18 pmol.

were precipitated with five volumes of methanol at – 20 °C overnight and then sedimented by centrifugation (15000 rpm, 30 min, at 4 °C). The sediment was dissolved in 0.1% SDS, reduced with mercaptoethanol and carboxymethylated.

Proteins were separated by SDS/PAGE and the proteolipid protein (PLP) band excised after Coomassie Blue staining. The gel piece was washed with water, and treated with trypsin in the appropriate buffer and the fragments isolated as described for myoglobin. The peptide mixture was separated by Tricine-SDS/PAGE (Fig. 7). The band marked by an arrow was applied to the gas phase sequencer for Edman degradation. The recordings of the first six HPLC analyses of the PTH derivatives of the tryptic fragment (Asn^{53} to Glu^{58}) of PLP are listed in Fig. 8.

Discussion

The most frequently used method for the isolation of separated protein or polypeptide bands from polyacrylamide gels is their electrotransfer to membranes of different surface coating and binding properties. The variables in the modification of this general principle of electroblotting are:

1) the properties of the protein or polypeptide to be transferred (size, hydrophobicity, hydrophilicity),
2) binding properties of the membrane,
3) transfer conditions (voltage settings, transfer buffer conditions, temperature, contact of membrane and gel, blotting time).

These are the critical points for the transfer results which are hardly transferable and reproducible from one protein to the other and therefore lead to very divergent recoveries from the polyacrylamide gels. In our hands proteolipid protein, a strongly hydrophobic myelin protein (31 kDa) could not be transferred under several conditions (0.1% SDS, changing the methanol concentration from 20 to 5%, pH from 7 to 10).

A procedure which would give a high – if not almost quantitative – recovery of proteins or polypeptides directly from the respective gel slices by direct elution with appropriate solvents should not only facilitate microsequencing but also speed up the analysis.

In comparison to other procedures, where additional steps (e.g. salt or detergent removal) are involved, we describe a new, rapid and convenient two-step method. In the first step the protein is eluted and at the same time a proteolytic or chemical cleavage is performed. In the second step the polypeptides are concentrated by rotary evaporation or SpeedVac and applied directly to a Tricine-SDS gel. No detergent or salt removal is necessary during this procedure.

The eluted peptides from the Tricine-SDS gel can directly be transferred to the gas phase sequencer even if they contain a SDS concentration of 6%. These high detergent and salt concentrations do not interfere with chemical reactions of the Edman degradation. The PTH amino acid HPLC chromatograms do not show any background, additional elution peaks or any baseline drifts.

The solvent extraction method described here was tested with three proteins of rather different physicochemical properties: the water-soluble whale myoglobin, the lysosomal sphingomyelinase requiring low concentrations of detergents for solubilization and the water-insoluble strongly hydrophobic proteolipid protein from bovine brain myelin.

Our method was applied to the cyanogen bromide or tryptic fragments separated by the high resolution Tricine SDS/PAGE.

The results of the microsequencing analyses were reproducible and the background observed was surprisingly low. Needless to say that a prerequisite of the method is the purity of the sample and rough quantitation which has to be assessed with scrutiny before starting the Edman degradation by gas phase sequencing.

Materials and Methods

The derivatized glass fiber membrane (siliconized glass fiber purchased from Biometra) was used for electroblotting in the Sarto blotting chamber IIS following the experimental details described by Eckerskorn et al.[5]. Electroblotting was extended to 2 h for myoglobin, 5 h for sphingomyelinase, and 2 to 12 h for proteolipid protein. An efficient contact between gel and membrane was guaranteed by a 2 kg weight on the cover of the blotting chamber.

To reduce the contamination of separated proteins and peptides to a minimum, only highly purified solvents and reagents (methanol, acetic and formic acid etc.) with at least p.a. or better HPLC grade quality were used.

Gel electrophoresis grade acrylamide and *N,N*-methylenebisacrylamide were purchased from Bio-Rad, München, sodium acetate (HPLC grade) from J.T. Baker, Phillipsburg, protein sequencing grade proteinases from Boehringer, Mannheim, p.a. SDS from Sigma, Deisenhofen. For all solutions, gel buffers, electrophoresis buffers etc. only highly purified water from a milli Q reagent water system (Millipore, Eschborn) was used. The protein elution, cleavage reactions, rotary evaporations were only done in silanized Sovirell glass tubes to reduce loss of protein due to hydrophobic interactions.

Determination of protein recovery and the efficiency of the elution

Protein concentrations recovered from the gel slices were estimated from the comparison of integrated PTH amino-acid peaks of the standard mixture and the first cycles of the microsequencing analysis. The completeness of the protein elution from the gel slices was ascertained by subsequent staining with silver nitrate[13].

Cyanogen bromide cleavage of whale myoglobin

Myoglobin (100 μg) was dissolved in 500 μl 70% formic acid with 500 μg BrCN and stored at room temperature in the dark overnight. The solution was dried by rotary evaporation and dissolved in SDS sample buffer (4% SDS, 100mM Tris/HCl pH 8.0, 2% β-mercaptoethanol, 10% glycerol and 0.1% bromphenol blue). The peptide mixture was separated electrophoretically on a Tricine-SDS/polyacrylamide gel (10.5% T, 3% C-system)[14].

For comparison lanes with the BrCN fragments were transferred from an unstained gel by semi-dry blotting to a glass-filter membrane (Biometra), stained with Coomassie Blue, bands were excised and used for sequencing.

Coomassie Blue-stained polypeptide bands of a second gel were excised, washed for 2 h with water. Peptides were eluted at 37 °C overnight with 0.1% SDS, 100mM sodium acetate pH 8.5 keeping the volume of the elution buffer as small as possible (between 100 and 600 μl). The eluate was concentrated in a Speed Vac if necessary and directly transferred for gas-phase sequencing.

Cyanogen bromide cleavage of human lysosomal sphingomyelinase

Human lysosomal sphingomyelinase was purified to homogeneity by using a set of affinity columns and a preparative SDS gel in the last purification step (unpublished results). The gel was stained for about 15 min with Coomassie Blue and destained (for about 15–20 min) until the protein bands were just visible. A short time of staining and destaining minimizes contamination of the proteins (especially with formaldehyde, an impurity of methanol) and increases the recovery of polypeptides from polyacrylamide gels. The gel slice with the sphingomyelinase 70-kDa band was excised and washed with water to remove methanol and acetic acid. The gel piece was covered with a solution of 70% formic acid, 0.1% SDS, 0.1% mercaptoethanol and 1 mg BrCN. The reaction was performed in a silanized Sovirell glass tube in the dark for 15 h at room temperature. The supernatant was concentrated in a rotary evaporator to remove the formic acid and residual BrCN. The solution should not be concentrated to dryness, since this often causes insolubility of the peptides. This has been observed with other proteins as well. The solution was therefore concentrated to 100–200 μl final volume, diluted with water to 500–1000 μl and again concentrated to approximately 100 μl to reduce residual formic acid and BrCN.

The concentrated solution was brought to 10% glycerol, 0.1% bromphenol blue and 1% mercaptoethanol, heated at 42 °C for 1 h and finally applied to a Tricine/SDS polyacrylamide gel[14].

Separation of peptides and sample preparation for sequencing

Human lysosomal sphingomyelinase was purified to homogeneity (unpublished results). Whale myoglobin was purchased from Sigma. Myelin was isolated according to Norton[12] and proteolipid protein purified from the chloroform/methanol extract (2:1 v/v) by preparative SDS-PAGE. Peptide mixtures were concentrated in a Speed Vac or by rotary evaporation to a volume of 50–100 μl. If the volume exceeds 600 μl a microdialysis in dialysis tubes with 1000 Da cut off or an ethanol precipitation at – 20 °C overnight was carried out to reduce the high salt or detergent concentration which would affect the subsequent PAGE. We used the Tricine-SDS/PAGE for the separation of peptides with high resolution in the range between 1 to 100 kDa[14]. Peptide bands were stained with Coomassie Blue R250 and cut out of the gel. The gel pieces were incubated in the smallest volume possible (< 600 μl) of 0.1M sodium acetate pH 8.5, 0.1% SDS overnight at 37 °C. The supernatant of up to 600 μl was directly applied to the filter disc of the sequencer successively in 30-μl aliquots and dried in the N_2 stream of the sequencer. Peptides in volumes larger than 600 μl are precipitated with ethanol as described above.

Protein sequence analysis

Sequence analysis was carried out by Edman degradation using the protein sequencer model 477A from Applied Biosystems. PTH amino acids were separated by microbore HPLC using a C_{18} microbore (2.1 mm) HPLC column (Applied Biosystems), a Brownlee HPLC pump, a Spectroflow 773 detector (Kratos division) and a pneumatically controlled Rheodyne injection valve, Type 7125 (Latek, Heidelberg). Integration of HPLC peaks were carried out by the Kontron Integration Pack 450 adapted to an IBM PC AT compatible. All peptide solutions were applied directly in 30-μl aliquots to the sample filter, dried under nitrogen in the reaction cartridge chamber of the sequencer. Larger volumes were applied up to 600 μl in aliquots. Samples should be dried for 5 min.

The standard Applied Biosystems "begin cycle" preceding the first Edman degradation cycle removed SDS and salts. Each Edman degradation cycle lasted 43 min separated by 15 min intervals for equilibration, the HPLC run for PTH amino-acid identification lasted 30 min.

This work was supported by the *Bundesministerium für Forschung und Technologie, Genzentrum Köln* and the *Deutsche Forschungsgemeinschaft*, Sonderforschungsbereich 243.

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