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# BLOTTING OF PROTEINS ONTO IMMOBILON MEMBRANES
## *IN SITU* CHARACTERIZATION AND COMPARISON WITH HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The electrophoretic transfer from polyacrylamide gels to Immobilon [poly(vinylidene difluoride)] membranes of various proteins differing in molecular masses from 14 000 to 200 000 was performed, using both a semi-dry blotting apparatus and a standard blotting chamber. The blotted proteins were analyzed and sequenced with and without staining, and the initial yields of the degradation were examined. Furthermore, protein purification by blotting after one- and two-dimensional gel electrophoresis was compared with conventional HPLC methods. Optimum blotting conditions for *in situ* enzymatic or chemical cleavages of the proteins on the blots are described, and for the *in situ* hydrolysis followed by amino acid analysis and cysteine determination.

## INTRODUCTION

Over the past 10 years, blotting procedures have become an essential element in the biochemical analysis of nucleic acids, proteins and lipids. Several articles have been published on various technical aspects of these methods, and different blotting procedures have been proposed as means for studying biological problems.

Blotting or transfer of electrophoretically resolved proteins to immobilizing matrices, followed by specific detection, has become a routine procedure for the biochemical characterization of these macromolecules. For optimum results the following criteria must be met: (i) proteins must be transferred efficiently from the gel to the matrix; (ii) they must be quantitatively bound; (iii) the detection must be specific and sensitive and (iv) the blotted substances must allow senstitive analysis and characterization by appropriate micro methods. The combined use of sodium dodecyl sulphate (SDS) with polyacrylamide gel electrophoresis (PAGE) and discontinuous buffer systems[1,2] allows evaluation of the purity or the complexity of protein mixtures, the resolution of which has been increased by the development of two-dimensional gel electrophoresis[3–5]. Further improvement was made when blot techniques for the analysis of DNA[6] were applied to proteins[7–9]. Recently, methods for protein microsequence analysis have been described which employ the electrotransfer of pro-

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teins onto sequencer-stable supports, such as modified glass fibres (GFs) or poly(vinylidene difluoride) (PVDF) membranes[10–15].

However, many proteins cannot be sequenced by Edman degradation, due to blockage of the $NH_2$-terminal amino acid. Furthermore, modified $NH_2$-terminal amino acids cannot easily be determined. Sequence information for these proteins is obtained by cleavage of the polypeptide chain and purification of the peptides derived. As has been reported[16,17], proteins blotted onto various membranes can be cleaved enzymatically or chemically, and the peptides released can be separated by reversed-phase high-performance liquid chromatography (HPLC).

In this paper, we describe the enzymatic digestions of standard proteins *in situ* on PVDF membranes under different conditions, including the use of organic solvents, which were reported[18] to lead to an efficient elution of the polypeptides from the membranes. The activity of trypsin under these conditions was tested and the initial yields from stained or non-stained protein blots were examined. Furthermore, the advantages and applications of protein blotting *versus* purification by reversed-phase HPLC methods for complex protein mixtures are demonstrated on examples of ribosomal protein mixtures.

## EXPERIMENTAL

### *Materials*

PVDF membranes (Immobilon Transfer) of pore size 0.45 μm were obtained from MilliGen (Division of Millipore, Bedford, MA, U.S.A.). Poly(vinylpyrrolidone) with an average $M_r$ of 40 000 (PVP-40) was obtained from Sigma Chemie (Deisenhofen, F.R.G.), polybrene from Applied Biosystems (Foster City, CA, U.S.A.), trypsin, TPCK (L-1-tosylamido-2-phenylethylchloromethyl ketone)-treated trypsin from Boehringer (Mannheim, F.R.G.), sodium dodecyl sulphate (SDS), N,N′-methylenediamine and ammonium persulphate from Bio-Rad (Heidelberg, F.R.G.), 2-mercaptoethanol, methanol, glycine, tris(hydroxymethyl)aminomethane (Tris), Amido Black, thioglycolic acid, hydrochloric acid and dithiothreitol from Merck (Darmstadt, F.R.G.). Unless stated otherwise, all chemicals were of analytical grade or better quality. The marker proteins were from Bethesda Research Laboratories (BRL) (Gaithersburg, MD, U.S.A.). Vydac column packings, $C_4$ (300 Å, 5 μm) and $C_{18}$ (201 Total Porous Beads), were obtained from the Separation Group (Hesperia, CA, U.S.A.). The columns were packed in our laboratory.

### *Methods*

*SDS-PAGE.* Samples of 500 pmol β-lactoglobulin B were loaded onto gels (16 cm × 10 cm), which were subjected to pre-electrophoresis overnight at 20 V prior to sample application. Thioglycolate (0.1 m*M*) was added to the buffer. The polyacrylamide concentration was 15%, and the gels were prepared according to Laemmli[1].

The gels used for blotting of standard proteins in the two different blotting chambers (the semi-dry and conventional tank buffer system described below) were 5-15% gradient gels or 10% discontinuous polyacrylamide gels and were prepared as described above. The electric potentials were 60 V for the stacking gel and 100 V for the separation gel.

*Electrophoretic transfer.* Two blotting systems were used: the semi-dry appara-

tus from H. Hölzel (Dorfen, F.R.G.) and the conventional blotting chamber of standard design[8] with an interelectrode distance of 10.5 cm.

For SDS gels, the transfer buffer in both blotting systems consisted of 25 m*M* Tris–HCl (pH 8.4)–0.5 m*M* dithioerythritol (DTE)–0.02% SDS and was degassed before use. Transfer for the semi-dry apparatus was conducted for 1–2.5 h at 0.5–0.8 mA/cm$^2$. Transfer with our conventional system[15] was conducted for 1 h at 120–150 mA and then for 4–6 h at 650 mA. For electroblotting from two-dimensional gels in urea, the composition of the transfer buffer was 1% acetic acid and 0.5 m*M* DTE. In this case, the blotting was conducted at 4°C for 1 h with 650 mA, using the conventional tank buffer system.

In all cases, two PVDF membranes were sandwiched for blotting. After transfer, the membranes were washed thrice with HPLC-grade water (MilliQ system of Millipore, Eschborn, F.R.G.) for *ca.* 10–15 min with *ca.* 20 ml water per 10 cm × 10 cm membrane. The protein bands were excised and either sequenced immediately or stored at −20°C under nitrogen in sealed Eppendorf tubes. In cases where the membranes were stained, they were treated either with 0.1% R-250 in 50% methanol for 5 min and destained in 50% methanol for 5–10 min at room temperature[13] or with Amido Black in 0.1% in 50% methanol for 5 min and destained in 30% methanol for 5–10 min at room temperature. The protein bands that were not stained were detected as grayish areas while the membrane became white upon drying.

*Sequencing of immobilized proteins.* The excised bands were arranged as pieces in a single layer on top of a Polybrene-treated glass fibre disk in the upper cartridge block of an Applied Biosystems (Weiterstadt, F.R.G.) Model 477A sequencer, equipped with a Model 120 phenylthiohydantoin (PTH)-amino acid analyzer. The glass-fibre filters were pre-treated with trifluoroacetic acid, loaded with 2 mg Polybrene, and twice precycled, as instructed by Applied Biosystems. Sequencing and analysis of PTH derivatives were performed by standard procedures. Initial coupling yields were determined from recovery of the $NH_2$-terminal residue and are presented with respect to the amount of sample loaded onto the gels.

*In situ tryptic digestion.* Tryptic digests were performed on dot blots of β-lactoglobulin on PVDF membranes, which were prepared as follows. Small pieces of a PVDF membrane were immersed in 100% methanol. Before the membranes became dry, 10 μl of β-lactoglobulin (1 nmol) were spotted onto it, and the membranes were dried overnight in open test-tubes.

Each membrane, containing 1 nmol protein, was incubated for 30 min at room temperature in 1 ml 0.2% PVP-40, dissolved in 100% methanol, in order to prevent adsorption of the protease on the PVDF membrane during digestion[16,17]. Prior to enzymatic digestion, the excess of poly(vinylpyrrolidone) (PVP-40) was removed by extensive washing with water, at least three times.

The protein samples were digested as follows: 100 m*M* N-methylmorpholine acetate buffer (pH 8.1) was used as the digestion buffer, and the digestion was performed for 20 h at 37°C with gentle stirring. The enzyme/protein ratio was 1:20. The peptides were separated by reversed-phase HPLC on a Vydac $C_{18}$ 201 TPB column (250 mm × 4 mm) with an acetonitrile gradient in 0.1% aqueous trifluoroacetic acid. A complete removal of PVP-40 prior to HPLC analysis is essential, because it absorbs strongly in the UV region.

*In situ chemical cleavage with CNBr.* A 500-pmol amount of β-lactoglobulin

was loaded onto SDS polyacrylamide gel, which was subjected to pre-electrophoresis. Electroblotting onto PVDF membranes was carried out by using the transfer buffer and the apparatus described above. After blotting, the membranes were washed, and after inspection the spots were excised and collected in a small test-tube. The protein was cleaved with CNBr in 70% formic acid with 2 μl β-mercaptoethanol (ME) for 48 h in the dark under nitrogen at ambient temperature or, alternatively, in 70% formic acid, 2 μl ME and 20% acetonitrile under the same conditions. A 200-μl volume of the solution was added per $cm^2$ of excised membrane. After cleavage, the solution was diluted in water, the membrane taken out and the solution was lyophilized. The peptides were dissolved in 20 μl water, separated by reversed-phase HPLC ($C_{18}$, 5-μm packing). Sequencing and analysis of the PTH derivatives released were performed by standard procedures.

*Total hydrolysis.* The membrane pieces containing blotted β-lactoglobulin or insulin B (oxidized) were immersed in 250 μl of 5.7 *M* HCl and hydrolyzed for 24 h at 110°C in precleaned, evacuated, sealed test-tubes. After hydrolysis, the membranes were removed and the hydrolysate was dried in a desiccator or in a Savant (München, F.R.G.) Speed-Vac centrifuge for about 2 h. The amino acids were determined after precolumn derivatization with *o*-phthaldialdehyde (OPA) by reversed-phase HPLC, as described in ref. 19.

*Cysteine determination.* Lysozyme, insulin B and ribonuclease (each 1 nmol), respectively, were electroblotted in the semi-dry apparatus described above. In this case, the SDS-PAGE pre-electrophoresis of the SDS-polyacrylamide gel was not used. After blotting, the protein bands were rinsed with methanol for about 1 s, collected in hydrolysis tubes and 200 μl 98% formic acid was added to each tube. A 1-ml volume of 98% formic acid was mixed with 0.1 ml $H_2O_2$ and kept at room temperature for about 1 h. After cooling to 4°C, 40 μl of the performic acid solution were added to each protein blot in 200 μl 98% formic acid, and oxidation at −20°C was carried out for 2–4 h. The excess of performic acid was destroyed by the addition of 400 μl ice–water. The solution was evaporated in the Savant Speed-Vac centrifuge. Cysteines were determined as cysteic acid after acid hydrolysis, as described above.

## RESULTS AND DISCUSSION

### *Electrophoretic transfer*

As described under Methods, the electrotransfer of high- and low-molecular-weight (200 000 and 14 000) proteins was performed in a tank of buffer[15] or in a semi-dry apparatus[20], employing two sandwiched PVDF membranes (see Fig. 1A). The transfer buffer in both systems was the same, containing 25 m*M* Tris (pH 8.4)–0.5 m*M* DTE–0.02% SDS[15]. However, different blotting times and electric potentials were used in the two devices (see Methods). The transfer buffer was prechilled, and blotting was conducted at 4°C. Fig. 2 shows the transfer of several proteins from a gradient of 5–15% PAGE, using the tank buffer[15] under the conditions described above. From this Figure it is obvious that proteins with relative low molecular masses, such as β-lactoglobulin (18 400) or lysozyme (14 300), are transferred faster than the high-molecular-weight components. They almost totally penetrate the first membrane and are trapped on the second PVDF layer. Three of the other proteins, with $M_r$ 29 000 (carbonic anhydrase), 43 000 (ovalbumin) and 68 000 (bovine serum albu-

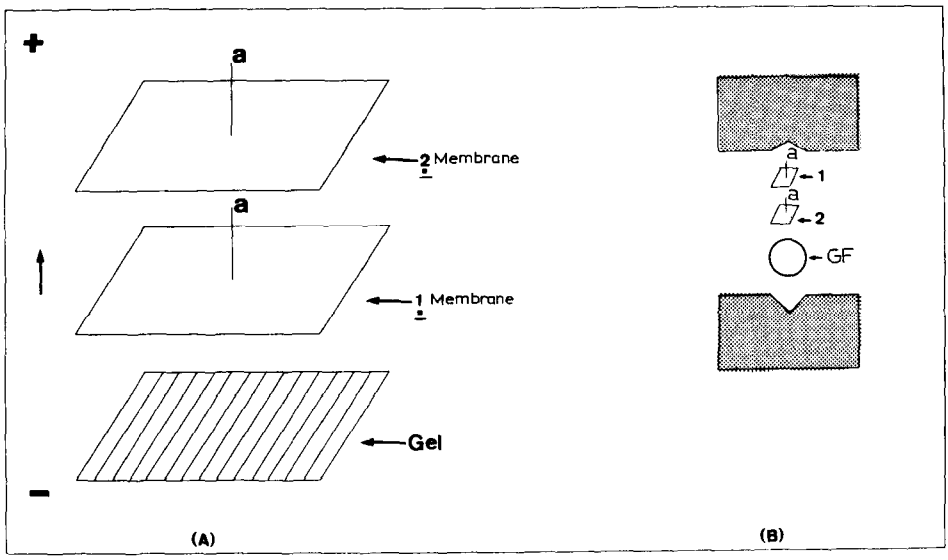



Fig. 1. (A) Electrotransfer from a SDS gel onto PVDF membranes. The arrow indicates the blot direction and "a" shows the membrane surface on which most of the protein was detected. (B) Arrangement of the PVDF pieces in the reactor. Numbers 1 and 2 show the first and second PVDF membrane, respectively; GF represents the glass-fibre disk, pre-treated with TFA, and precycled with Polybrene.

min), were partially transferred to the second PVDF layer. The high-molecular-weight components phosphorylase B and myosin (H-chain), with $M_r$ of 97 000 and 200 000, respectively, were transferred in only poor yields.

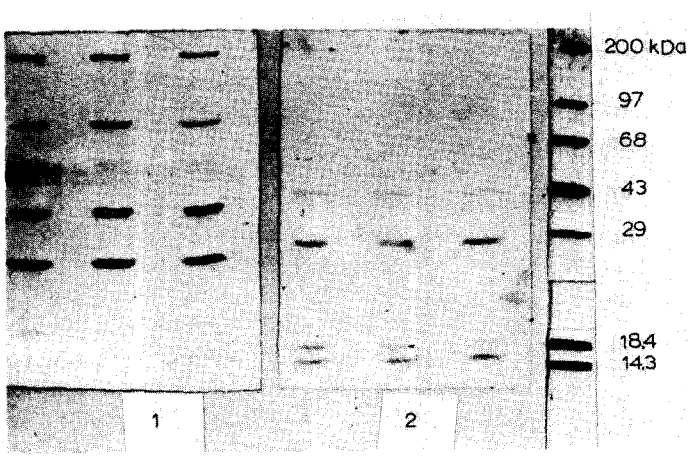



Fig. 2. Electrotransfer of various proteins onto PVDF membranes from a 5–15% gradient gel with the conventional tank buffer system. The transfer buffer consisted of 25 m*M* Tris (pH 8.4)–0.5 m*M* DTE–0.02% SDS. Numbers 1 and 2 represent the first and the second membrane, respectively. Blotting was conducted at 4°C, 150 mA for 1 h and 650 mA for 4 h. The membranes were weakly stained with Coomassie Blue for 5 min.

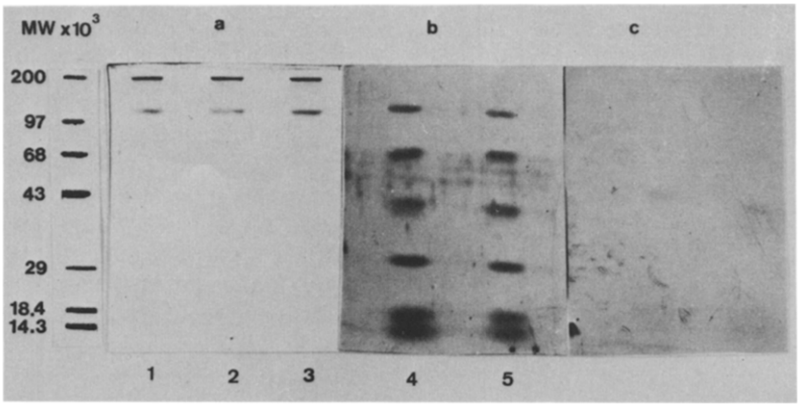



Fig. 3. Electrotransfer of proteins onto PVDF membranes from a 10% polyacrylamide gel by the semi-dry method. The transfer buffer was the same as in Fig. 2. Blotting was conducted at 4°C for 1 h with 0.8 mA/cm$^2$. The membranes were weakly stained with Coomassie Blue. "a" shows the remaining proteins in the gel, "b" and "c" the blotted proteins on the first and second membrane, respectively. Lanes: 1–4, loaded with the proteins described in Materials; 5, identical to 4.

Figs. 3 and 4 show the protein transfer from 10 and 5–15% PAGE, respectively, by the semi-dry method[20], which was used at 4°C for 1 h with 0.8 mA/cm$^2$. Problems with heating of the buffer during the transfer at 4°C were negligible under these conditions. Again, a poor transfer of the high-molecular-weight components (200 000 and 97 000) was observed, which necessitates the use of more SDS in the buffer system or a longer transfer time. On the other hand, the low-molecular-weight proteins were better transferred by using the homogeneous 10% gel which has bigger sizes of pores.

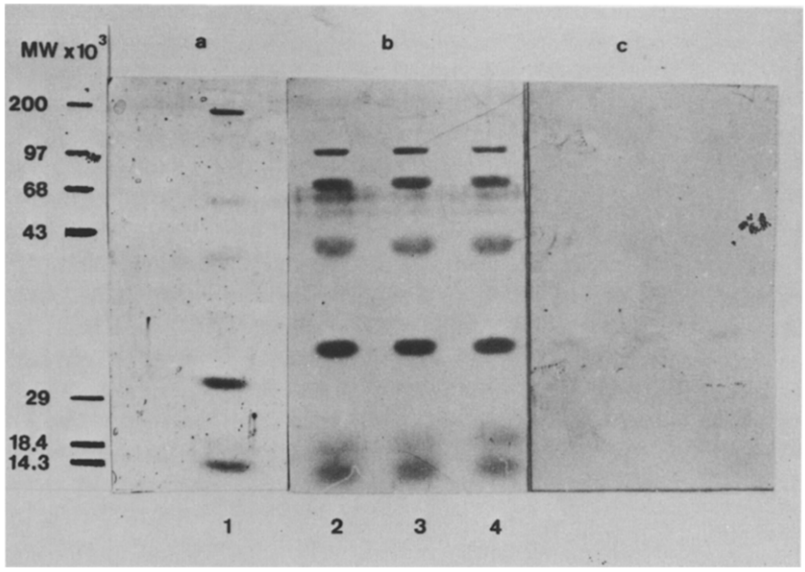



Fig. 4. Electrotransfer of proteins onto PVDF membranes from a 5–15% gradient gel by the semi-dry method. Blotting conditions and abbreviations as in Fig. 3.

Comparison of the transfers with the conventional tank system and the semi-dry apparatus shows that the lower-molecular-weight proteins blotted by the semi-dry method were transferred sufficiently and almost quantitatively to the first PVDF layer, independent of the size or charge of the proteins. These transfers, in which a flat electrode system with short distances between the electrodes and filter-paper reservoirs for the buffers, is used, offer advantages over the blotting in tanks in terms of homogeneous voltage fields and high voltage gradients, which produce rapid, efficient, safe and convenient electrotransfer. On the other hand, high-molecular-weight proteins need more time for the transfer, if the same transfer buffer is used. Therefore, for complex protein mixtures containing various sizes of proteins, such as ribosomal proteins, two layers of PVDF should be sandwiched to make sure that all the proteins can be trapped. Furthermore, an optimum transfer time must be found.

*Sequencing of immobilized protein*

A 500-pmol amount of β-lactoglobulin was loaded on a 15% polyacrylamide gel, which was subjected to pre-electrophoresis prior to protein application. The electrotransfer was conducted at 4°C in the conventional tank buffer system[15]. After transfer, the Immobilon membranes were quickly rinsed in water of HPLC grade for removal of glycinate ions, which interfere with sequencing, and for reduction of the SDS level, in order to prevent blockage of the HPLC injection line in the sequencer. The proteins were detected by weak staining with Coomassie Blue or Amido Black. They can also be detected without staining as grayish areas on the surrounding white membrane during drying. With further drying, the protein-containing areas are easily seen as zones more intensely white than the surrounding ones when transilluminated with white light. The bands were excised with a razor blade, dried overnight at room temperature and stored in sealed Eppendorf tubes under nitrogen at −20°C.

The arrangement of the Immobilon pieces in the reactor is shown in Fig. 1B. At first, the membrane surface onto which the protein is blotted (showing the highest intensity) is placed in the upper cartridge in upside-down position. Secondly, the second PVDF membrane piece is placed on top of the first one, and thirdly, both are kept in place by covering them with a trifluoroacetic acid (TFA)-treated glass filter, pretreated with 2 mg Polybrene, and precycled in a normal sequencing program. The upper cartridge is then turned over and screwed on to the lower cartridge part, so that the "protein face" is on top. At the $R_3$ delivery and wash stages, the blotted protein is transferred and trapped on the pretreated glass filter. Thus, the blotted proteins or peptides are degraded by this technique mainly in the glass filter, and incomplete coupling due to insufficient penetration of the aqueous base into the PVDF membrane pieces is prevented. Table I shows the difference between blotted unstained β-lactoglobulin samples, which were arranged either with the "protein face" in the cartridge on top as described above, or randomly arranged. The initial yields of 40% in the first case and 21.8% in the second case demonstrate that the correct placement of the protein side of the PVDF membranes is very important.

In order to demonstrate that blotted proteins easily become $NH_2$-terminally blocked, not only during electrophoresis and electrotransfer[21], but also during staining, even if all the chemicals used are of high-purity grade, we sequenced 500 pmol of blotted β-lactoglobulin, either stained with Coomassie Blue or Amido Black or unstained, using identical conditions for electrophoresis, transfer and sequencing (see

TABLE I

EFFECT OF THE POSITION OF THE PROTEIN BLOTS IN THE CARTRIDGE BLOCK ON INITIAL SEQUENCING YIELDS (SEE FIG. 1B)

β-Lactoglobulin samples (500 pmol) were applied onto the gel and blotted after electrophoresis. Initial yields were determined for the $NH_2$-terminal residues. The values shown are the percentages of the amounts of protein loaded onto the gel. The yields are average values from three experiments.

| *Detection* | *Arrangement* | *Yield (%)* |
|---|---|---|
| Coomassie Blue[a] | Protein face[b] | 24.1 |
| Amido Black[a] | Protein face | 15.0 |
| Non-stained | Protein face | 40.0 |
| Non-stained | Random | 21.8 |

[a] Weakly staining for 5 min.
[b] The "protein face" arrangement is described in Fig. 1B and in the text.

Table I). The Coomassie-stained samples gave 24.1% initial yield, and the Amido Black-stained yielded 15.0%, while the non-stained samples gave 40% for the "protein face" arrangement and 21.8% for a random arrangement.

*Cleavage with CNBr*

The cyanogen bromide cleavage *in situ* on the Immobilon was used to obtain internal sequences of known blocked proteins. The mixture of the peptides was dissolved in about 20–30 μl water, then applied on a TFA-pretreated glass filter, and sequenced according to the standard programs. For cleavage of small polypeptides with a $M_r$ of about 6000, addition of acetonitrile to the cleavage solution was unnecessary, whereas for cleavage of larger proteins, such as β-lactoglobulin (18 400), this was very useful (final concentration, 20% acetonitrile). The presence of an organic solvent helps to wet the PVDF membrane, facilitating cleavage of the protein and extraction of the peptides from the membrane. The use of Triton X-100 or other detergents was found unnecessary in case of the proteins tested.

Employing this technique, we studied a fragment of ribosomal protein L12 from *Sulfolobus acidocaldarius*, produced during autolysis of the protein. Since the intact L12 protein is $NH_2$ -terminally blocked (by acetylmethionine)[22], this fragment with a molecular mass of about 6000 was isolated by SDS-PAGE. The gel was subjected to pre-electrophoresis, and the fragment (1–2 nmol) was blotted in the tank buffer system. After *in situ* cyanogen bromide cleavage of the fragment on the PVDF membrane, we were able to determine the sequence of 58 residues[23]. This cleavage was performed without the addition of acetonitrile.

After chemical cleavage of a protein, *e.g.*, by CNBr *in situ*, on the blot, it is very important to dilute the generated peptide solution in water and to dry it several times. Multiple washing and drying removes the excess of CNBr and reaction products, which could block the delivery and injection lines of the sequencer.

*Cysteine determination*

The cysteine content was estimated for insulin B and for a ribosomal protein of *Bacillus stearothermophilus*. The blots were stained with Coomassie Blue and Amido

Black. The amount of protein loaded onto the gel was 2 and 1 nmol, respectively. The ribosomal protein was blotted from two-dimensional gels[5] by using the conventional tank buffer system[15]. The number of cysteines found for blotted oxidized lysozyme and ribonuclease was lower than that calculated from their sequences. Four cysteines were found for ribonuclease (theoretical value 8.0) and five for lysozyme (theoretical value 8.0). In part, this is to be expected after oxidation and hydrolysis. Likely, cysteine residues in blotted proteins need a longer oxidation time, and they are partially destroyed during electrophoresis and transfer. On the other hand, proteins with low molecular weights, such as insulin B (3400) and the ribosomal protein (*ca.* 6000), gave better results. The number of cysteines determined for the blotted insulin B was 1.67, and for the non-blotted protein it was 2.0. This is in good agreement with the number of cysteine residues present (two residues) in this polypeptide chain.

*Tryptic digestion*

The ability to perform *in situ* chemical or enzymatic cleavage of proteins blotted on PVDF membranes, followed by reversed-phase HPLC of the peptide fragments released, obviates many problems. In order to prevent adsorption of the protease on the membrane during digestion, the blotted pieces were pretreated with PVP-40, as described in ref. 16, with some modifications. We used a concentration of 0.2% in 100% methanol (see Methods). The cleavage was performed with an enzyme/protein ratio of 1:20, at 37°C in 100 m*M* N-methylmorpholine acetate buffer at pH 8.1.

Fig. 5a shows the HPLC separation of the tryptic peptides released from 1 nmol β-lactoglobulin without blotting, and Fig. 5b shows the separation of the same peptides produced *in situ* after gel electrophoresis and blotting. Fig. 6a and b illustrate the HPLC separation of the tryptic digest made in 100 m*M* N-methylmorpholine acetate buffer (pH 8.1) with 20% methanol and 10% acetonitrile, respectively. According to the results of these experiments, the enzymatic cleavage is optimal under the conditions used in Fig. 5b, with the N-methylmorpholine buffer and without addition of any organic solvent. Under the cleavage conditions described in ref. 16, we obtained a smaller release of fragments. Obviously, the activity of the enzyme is decreased more by the presence of acetonitrile than by methanol, and pretreatment of the membrane with PVP-40 is superior. (See Experimental.)

*Comparison HPLC versus blotting*

HPLC, mainly reversed-phase chromatography, is highly suitable for rapid purification of minute amounts of protein. Very complex protein mixtures are well resolved, *e.g.*, the many proteins extracted from ribosomal particles[24]. Furthermore, reversed-phase HPLC is frequently applied to peptide mixtures derived from enzymatic or chemical fragmentation of such proteins, where 20–30 peptides are generated. Other advantages of HPLC are that volatile buffers can be used with the addition of preservatives for protection of the free $NH_2$-terminal amino groups. This allows direct microsequencing and amino acid analysis of the dried peptide and protein fractions.

However, the usual high recoveries of peptides and proteins by reversed-phase HPLC decrease drastically with increased size and hydrophobicity of the proteins and drop further with acidic hydrophobic proteins, which are poorly resolved[15,25]. Proteins of $M_r$ greater than 30 000 are usually trapped on top of the column. Losses of

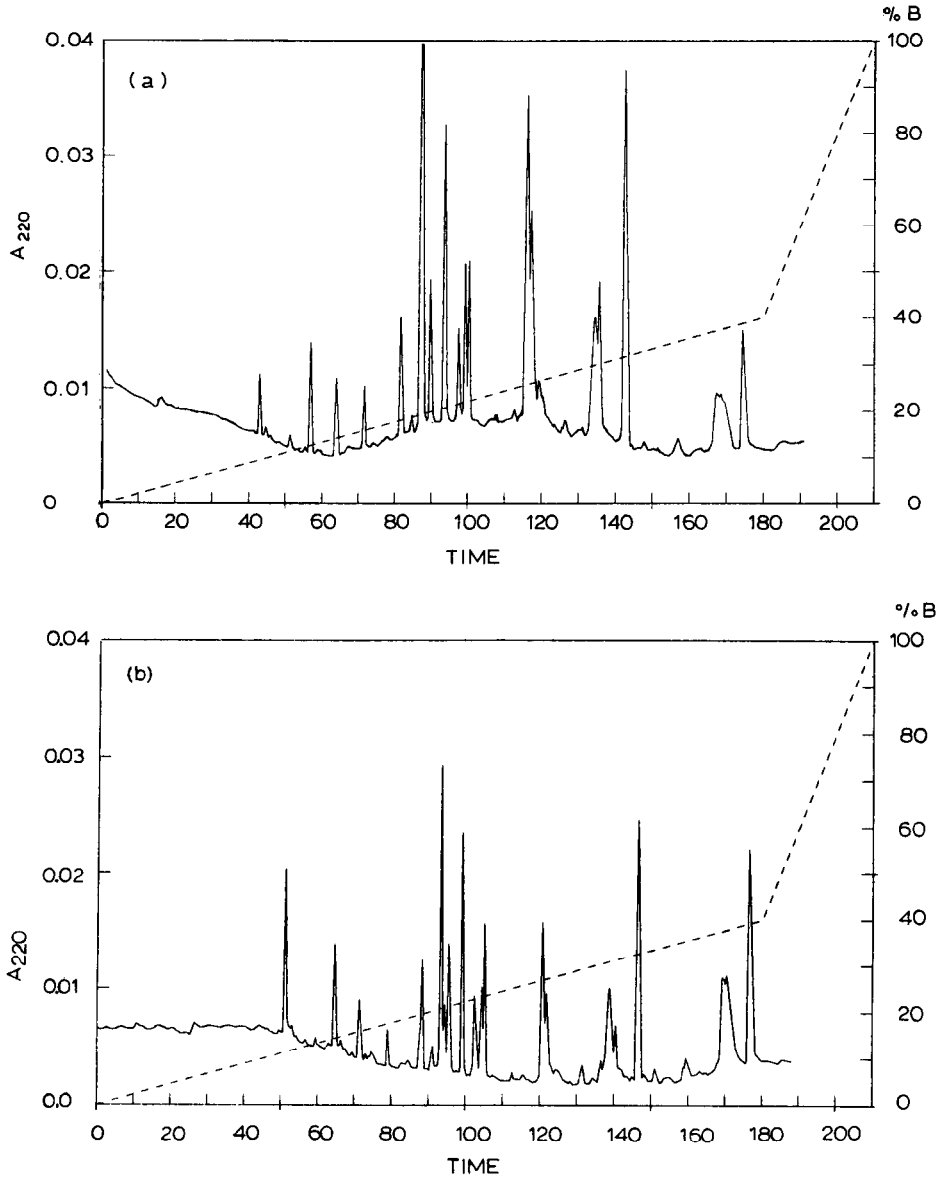



Fig. 5. (a) Tryptic peptides released from 1 nmol of β-lactoglobulin (without blotting) separated by HPLC on a Vydac $C_{18}$ column (250 mm × 4 mm). Eluents: A, 0.1% TFA; B, acetonitrile containing 0.1% TFA; flow-rate, 0.5 ml/min. Paper speed: 2 mm/min. Sensitivity: 0.04 a.u.f.s. The digestion took place in 100 m*M* aqueous N-methylmorpholine acetate (pH 8.1) at 37°C for 20 h. (b) Separation of tryptic peptides, produced by *in situ* digestion of 1 nmol blotted β-lactoglobulin under te same conditions as in (a). The PVDF membrane was treated with PVP-40, as described in Methods. The peptides released were separated by HPLC under the same conditions as in (a).

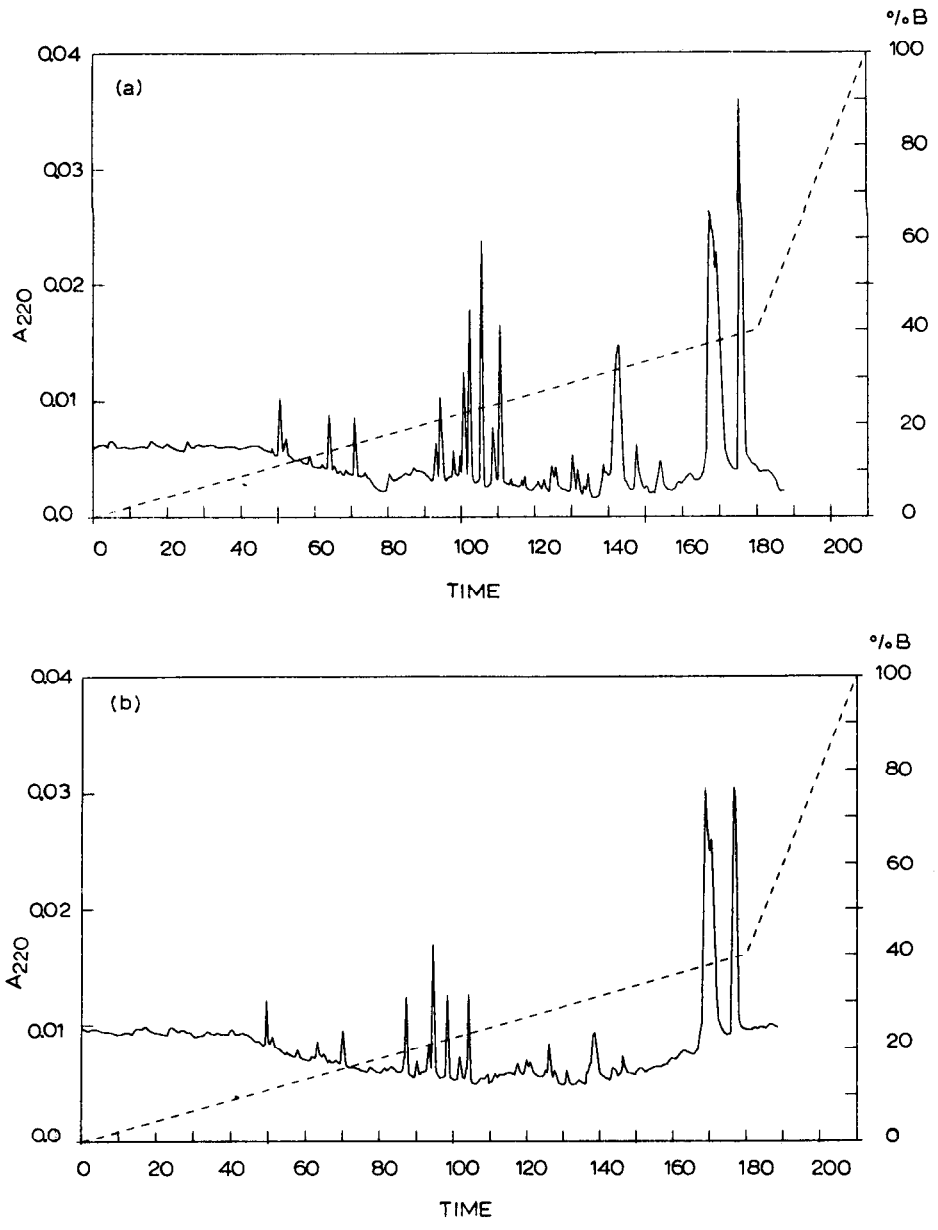
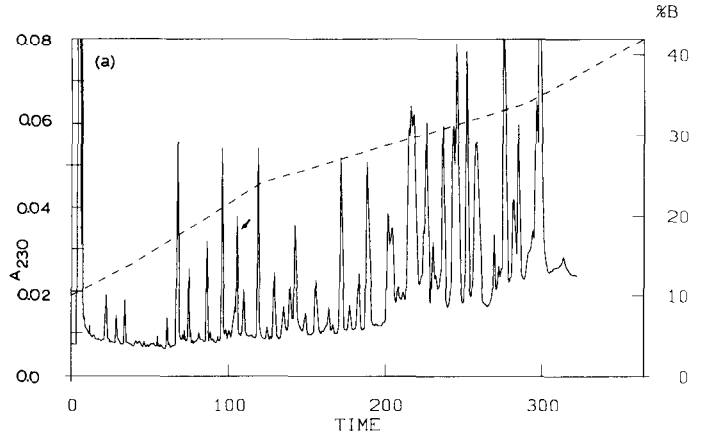



Fig. 6. (a) Separation of tryptic peptides produced by *in situ* digestion of 1 nmol blotted β-lactoglobulin. Digestion conditions as in Fig. 5, but the buffer contained 20% methanol in 100 m*M* N-methylmorpholine acetate at pH 8.1. The peptides were separated by HPLC, as in Fig. 5a. (b) Separation of tryptic peptides derived by *in situ* digestion of 1 nmol blotted β-lactoglobulin. Digestion conditions as in Fig. 5, but the digestion buffer contained 10% acetonitrile in 100 m*M* N-methylmorpholine acetate (pH 8.1). HPLC was performed as in Fig. 5a.

precious material might also occur during processing of the HPLC fractions, or due to dilution of the sample. In addition, problems are encountered due to variations in sorbents. Different batches and products from different manufacturers may show changes in the elution of diverse proteins.

Although the chromatograms often yield sharp and distinct peaks, the proteins may be contaminated with each other (*cf.*, *e.g.*, Fig. 7a and b). As an example, in Fig. 7a the protein indicated by an arrow is still not purified (*cf.*, Fig. 7b). Therefore, the purity of protein-containing fractions must be tested by rechromatography or by one- and two-dimensional gel electrophoresis. These disadvantages of HPLC are circumvented by the two-dimensional gel electrophoresis separation methods, which have an



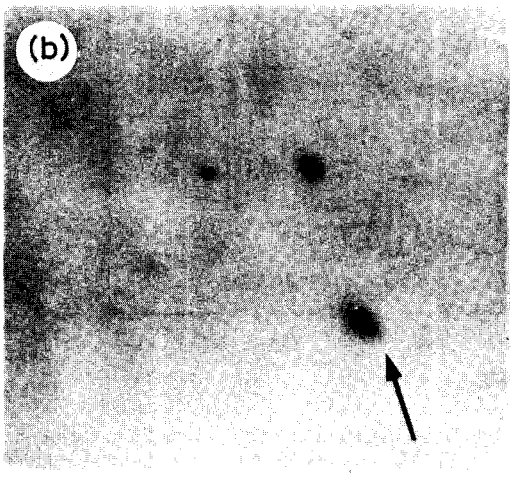




Fig. 7. (a) Separation of the protein mixture (2.7 mg) extracted from *Bacillus stearothermophilus* 50S ribosomes by reversed-phase HPLC on a Vydac $C_4$ column (250 mm × 4 mm) with a 2-propanol gradient in 0.1% TFA. Flow-rate: 0.5 ml/min. Paper speed: 1 mm/min. Sensitivity: 0.16 a.u.f.s. (b) Blotted proteins after two-dimensional gel electrophoresis of the peak in (a) indicated by an arrow. This peak contains one main and three minor components.

extremely high resolving power for complex protein mixtures[4] and are easy to perform without sophisticated equipment. In combination with protein blotting from these gels onto membranes suitable for sequencing, these methods have recently become an excellent alternative to HPLC. The blotting procedures complement HPLC in cases where the latter gives low resolution and low recoveries or where high-molecular-weight proteins are present in the mixture. Sequencing of the blotted proteins can successfully be applied provided that high-quality chemicals are used for gel electrophoresis and that necessary precautions to prevent blockage or destruction of labile amino acids are taken[15].

In this article, we have described transfer conditions that allow blotting of low- and high-molecular-weight proteins in the same experiment. Without removing the proteins from the membrane, direct microsequencing, enzymatic or chemical fragmentation or hydrolysis for amino acid analysis can be carried out. This procedure, especially when combined with two-dimensional gel electrophoresis, is a very powerful and efficient method for isolation and microsequencing of proteins in complex protein mixtures and may replace HPLC techniques.

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