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Strategies for internal amino acid sequence analysis of proteins separated by polyacrylamide gel electrophoresis

LARRY D. WARD, GAVIN E. REID, ROBERT L. MORITZ and RICHARD J. SIMPSON*
Joint Protein Structure Laboratory, Ludwig Institute for Cancer Research (Melbourne Branch), and the Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3050 (Australia)
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ABSTRACT

An evaluation has been made of various stategies for obtaining internal amino acid sequence data from electrophoretically separated proteins. Electroblotting, *in situ* proteolysis and extraction, and direct electroelution are compared. Electroblotting of protein or peptides from gels resulted in poor yields (typically, 1–7%). However, higher yields (3–67%) were achieved by *in situ* enzymatic cleavage followed by acid extraction of the peptides from the gel. Peptides extracted from the gel were separated by reversed-phase high-performance liquid chromatography (RP-HPLC), on short, small-bore columns (100 × 2.1 mm I.D.), to enable recovery of peptides in small volumes (*ca.* 50 μl) suitable for microsequence analysis. Capillary zone electrophoresis under acidic conditions (pH 2.5) was used to assess peptide purity before sequence analysis. Cysteine residues were identified in unmodified proteins or peptides by a characteristic phenylthiohydantoin (PTH)-amino acid derivative during sequence analysis. This derivative does not co-chromatograph with any known PTH-amino acid.

Direct electrophoretic elution of protein from gels yielded between 45–50% of applied protein. Proteins recovered from gels by electrophoretic elution required further purification by inverse-gradient RP-HPLC [R. J. Simpson, R. L. Moritz, E. C. Nice and B. Grego, *Eur. J. Biochem.*, 165 (1987) 21] to remove sodium dodecylsulphate and acrylamide-related contaminants for sequence analysis.

INTRODUCTION

Polyacrylamide gel electrophoresis (PAGE) is a simple, high-resolving technique for fractionating complex protein mixtures. Until recently, several technical difficulties have prevented the use of PAGE for preparative purposes, namely, the limited capacity for high-resolution separations (low microgram amounts of protein) and the inherent difficulties associated with recovering proteins from the gel matrix.

Traditionally, electroclution and passive elution have been the methods of choice for recovering proteins from gels [1–3]. However, proteins recovered by these means are typically contaminated with large amounts of sodium dodecylsulphate (SDS), buffer salts and acrylamide gel related contaminants which severely interfere with protein sequence analysis and subsequent chromatographic separation of phenylthiohydantoin (PTH)-amino acids. Attempts to recover protein from electro-eluates by selective precipitation with organic solvent (*e.g.* 90% methanol at −20°C), ion-pairing agents or organic solvent mixtures are often associated with significant losses of protein, especially when working at low microgram levels [1,2]. Recently, we

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reported an "inverse-gradient" reversed-phase high-performance liquid chromatographic (RP-HPLC) procedure for the efficient recovery of proteins from SDS-PAGE electroeluates [4–7]. This technique is based upon the skewed U-shaped (or bimodal) dependency that exists between retention times and concentration of organic solvent during the RP-HPLC of some small organic compounds, peptides and proteins on particular reversed-phase supports [4,8–11]. Typically, proteins are retained on small-pore-size (60–120 Å) reversed-phase packings at high concentrations of organic solvent (> 85%); under these conditions SDS and acrylamide gel related artifacts wash through the column. Retained proteins can be recovered in high yield (> 85%) from the column by the addition of trifluoroacetic acid [0.4% (v/v)] to the mobile phase and elution with a gradient of decreasing concentration of organic solvent (*i.e.* an "inverse-gradient").

An alternative approach for recovering proteins from polyacrylamide gels is by electrotransfer onto immobilizing membranes [12–14]. Provided that immobilizing matrices are chemically inert to the reagents and solvents used in the Edman degradation procedure, this approach constitutes an ideal way of preparing samples for N-terminal microsequence analysis. Several types of membranes have been used in this respect; these include derivatized glass fiber sheets [15–19] and polyvinylidene difluoride membrane (PVDF) [20,21]. (For recent reviews, see references 1, 2 and 21.)

Although N-terminal amino acid sequence analysis is often an important first step in establishing the identity of a protein, the complete amino acid sequence determination using recombinant DNA methodologies is now the preferred approach for an initial description of the complete sequence. Internal amino acid sequence data are invaluable for allowing the most efficient use of the molecular biological approaches (*e.g.*, the polymerase chain reaction [22]) for gene cloning. For N-terminally blocked proteins internal sequence data are mandatory for protein identification.

Recently, we reported a three-step procedure for obtaining internal amino acid sequence data for proteins electroblotted onto PVDF membranes [5]. At first, Coomassie blue-stained proteins were eluted from PVDF membranes using a detergent mixture of SDS and Triton X-100. In the second step, proteins were recovered from the detergent mixture by our previously reported "inverse-gradient" RP-HPLC procedure [4]. In the third step, proteins recovered by inverse-gradient RP-HPLC were subjected to proteolytic digestion and the generated peptides purified by microbore column RP-HPLC [1,23–25] for sequence analysis. However, in our experience [5,26] and that of others [27,28], the efficiency of electrotransfer of proteins from SDS-PAGE gels onto PVDF membranes is very low (in the range 28–30%). For this reason, alternative methods for obtaining internal sequence data from electrophoretically separated proteins, particularly those that do not require an electroblotting step are of obvious importance.

In this paper we compare the above-mentioned procedure for obtaining internal amino acid sequence data from gel-separated proteins with various other strategies. These strategies include: (i) *in situ* enzymatic digestion of protein in the gel matrix, separation of derived fragments by one-dimensional SDS-PAGE followed by peptide recovery from the gel by electroblotting; (ii) *in situ* enzymatic digestion of protein in the gel matrix, acid extraction of derived peptides from the gel matrix followed by separation of peptides by microbore column RP-HPLC; and (iii) direct electrophoretic elution of proteins from the acrylamide gel. Protein is recovered from the electroeluate

by inverse-gradient RP-HPLC, proteolytically digested and the derived peptides purified by RP-HPLC. The advantages and disadvantages of these methods are discussed.

MATERIALS AND METHODS

High-performance liquid chromatography

Instrumentation. The chromatographic equipment consisted of a Hewlett-Packard (Waldbronn, F.R.G.) liquid chromatograph (HP 1090M), equipped with an autosampler and diode-array detector (HP 1040A). Spectral and chromatographic data were stored on hard disc using a Hewlett-Packard chromatographic workstation. Manual injections were performed with a Rheodyne Model 7125 injector, equipped with a 2-ml injection loop, installed in the column oven compartment.

Column supports. The following packing materials, obtained from Applied Biosystems (Foster City, CA, U.S.A.), were used in this study: (a) Brownlee RP-300 (7 μm dimethyloctyl silica, 30 nm pore size, packed into a 100 × 2.1 mm I.D. cartridge) and (b) Brownlee VeloSep octyl (C_8) cartridge (3 μm octyl silica, 10 nm pore size, packed into a 40 × 3.2 mm I.D. cartridge).

Chemicals and reagents. β-Lactoglobulin, tris(hydroxymethyl)aminomethane (Tris) base, 3(cyclohexylamino)-1-propane sulfonic acid (CAPS), pepsin, thioglycolic acid and 2-mercaptoethanol were purchased from Sigma (St. Louis, MO, U.S.A.). TPCK-treated trypsin was from Worthington (Freehold, NJ, U.S.A.). Dithiothreitol was from Calbiochem (San Diego, CA, U.S.A.). Recombinant murine interleukin-6 (IL-6) was prepared as described elsewhere [29]. HPLC-grade organic solvents were purchased from Mallinckrodt (Melbourne, Australia). Trifluoroacetic acid (99+% grade) and Tween 20 were obtained from Pierce (Rockford, IL, U.S.A.). Acrylamide, methylene bisacrylamide, glycine and urea were from BioRad (Richmond, CA, U.S.A.). Deionized water, obtained from a tandem Milli-RO and Milli-Q system (Millipore, Bedford, MA, U.S.A.) was used for all buffers. Coomassie blue R-250 and sodium dodecyl sulphate (SDS) were purchased from LKB (Bromma, Sweden). PVDF membrane (Immobilon™) was obtained from Millipore. *Staphylococcus aureus* strain V8 protease was from Miles (Naperville, IL, U.S.A.).

Electrophoretic techniques

SDS-PAGE. Proteins and large peptides were separated on 12.5% polyacrylamide slab gels (1.5 mm thick and 16 cm in length) in a Protean II (BioRad) electrophoresis system using the Laemmli [30] discontinuous buffer system. Electrophoresis was performed at 25 mA constant current and running buffer was maintained at 4°C by circulation of water from an external cooling system. Gels were pre-electrophoresed with 0.375 *M* Tris–HCl buffer (pH 8.8) containing 0.1% (w/v) SDS, 1 m*M* thioglycolic acid for four hours at 60 V. Thioglycolic acid (1 m*M*) was included in the running buffer. After electrophoresis, gel proteins were either stained with 0.1% (w/v) Coomassie blue R-250 [in 50% (v/v) methanol, 10% (v/v) acetic acid] and destained with 12% methanol, 7% acetic acid or electrotransferred onto PVDF membranes.

Electrotransfer onto PVDF membrane. After electrophoresis, gels were equilibrated in electrotransfer buffer [10 m*M* CAPS buffer (pH 11.0) containing 10% (v/v)

methanol, 1 m*M* thioglycolic acid] for 15 min at 25°C. Electrotransfer was performed in a BioRad Transblot cell at 90 V for two hours at 4°C. Prior to use, PVDF membranes were rinsed in 100% methanol, water (5 min) and then electrotransfer buffer (15 min). Proteins were visualized by staining with 0.1% (w/v) Coomassie blue R-250 in 50% (v/v) methanol, 10% (v/v) acetic acid for 5 min and destained with 50% methanol and 10% acetic acid. After staining, PVDF membranes were washed thoroughly with water (5 min), air-dried for 20 min and stored at −20°C. To reduce background levels of PTH-amino acid derivatives during sequence analysis, PVDF membranes were washed with water overnight.

Direct electrophoretic elution

Direct electrophoretic elution from SDS-PAGE tube gels was performed using an Applied Biosystems Model 230A (HPEC™) electrophoresis apparatus. Proteins were separated on 8% polyacrylamide gels (50 mm long and 2.5 mm in diameter) using 25 m*M* Tris, 192 m*M* glycine, 0.1% SDS, pH 8.3 as cathode buffer and 25 m*M* Tris–HCl, pH 8.3 as both anode and elution buffers. Electrode buffers were changed at the rate of 1.5 ml/min to avoid polarization and build-up of electrode decomposition products. Elution buffer flow-rate was 12 μl/min. Gels were pre-electrophoresed for two hours at 170 V prior to application of sample. Samples were applied in volumes of 10 μl in 62.5 m*M* Tris–HCl buffer (pH 6.8) containing 3% SDS, 10% glycerol and 5% β-mercaptoethanol. Electrophoresis was performed at 0.8 mA constant current. Eluted proteins were detected on-line by absorbance at 280 nm and 60-μl fractions were collected automatically.

Capillary zone electrophoresis

Capillary zone electrophoresis (CZE) was performed using an Applied Biosystems capillary electrophoresis system (Model 270A) fitted with 122 cm × 50 μm I.D. capillary tubing (100 cm to the detector). The capillary tubing was prewashed with 0.1 *M* NaOH for 5 min prior to sample injection. Samples (*ca.* 0.01 mg/ml) in trifluoroacetic acid–acetonitrile were applied to the capillary under vacuum for 5 s. A standard peptide, Arg-Lys-Arg-Ser-Arg-Lys-Glu, used as a mobility marker, was introduced under vacuum for 3 s. Free solution electrophoresis was performed at 30 kV (20 μA) for 50 min using 20 m*M* sodium citrate (pH 2.5) as electrolyte and the capillary oven temperature was maintained at 30°C.

Elution of proteins from PVDF membranes

Coomassie blue-stained proteins were eluted from PVDF membranes with an aqueous detergent mixture [2% (w/v) SDS–1% (w/v) Triton X-100–0.1% (w/v) dithiothreitol] as described elsewhere [5].

Chromatographic recovery of proteins from detergent mixtures by "inverse-gradient" RP-HPLC

PVDF-eluted proteins were recovered from the detergent mixture by an "inverse-gradient" RP-HPLC procedure described elsewhere [4,5]. Briefly, the aqueous detergent eluate (*ca.* 200 μl) containing *ca.* 6 μg of Coomassie blue-stained protein was diluted in a glass gas-tight sample-loading syringe to *ca.* 1.5 ml with 1-propanol. The sample was applied, at 1 ml/min, to a VeloSep C_8 cartridge (40 × 3.2

mm I.D.) previously equilibrated (40 ml) with 90% (v/v) aq. 1-propanol. The column was developed at 200 μl/min with a linear 10-min gradient from 100% A to 100% B where eluent A was 90% (v/v) aq. 1-propanol and eluent B was 50% (v/v) aq. 1-propanol containing 0.4% (v/v) trifluoroacetic acid. The column temperature was 40°C.

Microbore column RP-HPLC purification of peptides

Peptides were separated on a Brownlee RP-300 column (30 nm pore size, 10 μm dimethyloctylsilica packed into a column (100 mm × 2.1 mm I.D.). The column was developed with a linear 60-min gradient from 100% A to 100% B, where solvent A was 0.1% (v/v) aq. trifluoroacetic acid and solvent B was 60% acetonitrile–40% water containing 0.09% (v/v) trifluoroacetic acid. Flow-rate was 100 μl/min; column temperature was 45°C.

Peptide mapping

In this study, three different methods for generating peptide maps of gel-separated proteins were examined.

Method 1: Proteolytic digestion of detergent-eluted electroblotted proteins followed by microbore column RP-HPLC separation of peptides. β-Lactoglobulin (20 μg) was electrophoresed (SDS-PAGE) on a 12.5% polyacrylamide gel and electrotransferred onto a PVDF membrane as described above. The electroblotted β-lactoglobulin was visualized with Coomassie blue R-250, excised and placed in a polypropylene (Eppendorf) tube. Protein was eluted from the PVDF membrane by soaking (twice) with 100 μl of 50 m*M* Tris–HCl buffer (pH 9.0) containing 2% (w/v) SDS, 1% (v/v) Triton X-100 and 0.1% (w/v) dithiothreitol at 25°C for four hours. The combined detergent eluates (200 μl) containing 5–10 μg of Coomassie blue-stained protein were diluted in a sample-loading syringe to 1.5 ml with 1-propanol and the protein recovered by "inverse-gradient" RP-HPLC [4,5]. β-Lactoglobulin-containing fractions [*ca.* 6 μg β-lactoglobulin in 300 μl 70% aq. 1-propanol containing 0.2% (v/v) trifluoroacetic acid, 0.02% (w/v) Tween 20] from "inverse-gradient" RP-HPLC were concentrated three-fold in a centrifugal vacuum concentrator (Savant, Hicksville, NY, U.S.A.) and then diluted to 1 ml with 5% (v/v) formic acid containing 0.02% (w/v) Tween 20. Digestion with pepsin (Sigma) was performed at an enzyme-to-substrate mass ratio of 1:20 at 37°C for one hour. Peptic peptides of β-lactoglobulin were purified for sequence analysis by microbore RP-HPLC [1,23–25] as described above.

Method 2: In situ proteolytic digestion of protein in gel slices followed by electrophoretic separation of peptides. Electrophoretically separated proteins were subjected to *in situ* enzymatic cleavage essentially as described by Cleveland [31]. Briefly, acrylamide gels were stained with 0.1% (w/v) Coomassie blue R-250 in 50% methanol–10% acetic acid (v/v) for 20 min and then destained in 7% acetic acid–12% methanol (v/v). Protein bands were excised, soaked for one hour in equilibration buffer [125 m*M* Tris–HCl buffer (pH 6.8) containing 10% (v/v) glycerol, 50 m*M* β-mercaptoethanol, 1 m*M* EDTA, 0.1% (w/v) SDS] and then positioned in the sample well of a second 15% polyacrylamide gel. Gel slices were overlayed with 50 μl of 125 m*M* Tris–HCl buffer (pH 6.8) containing 20% (v/v) glycerol, 1 m*M* EDTA, 50 m*M* β-mercaptoethanol and 0.1% (w/v) SDS followed by 2.5 μg *Staphylococcus aureus* strain V8 protease in equilibration buffer. A modified Laemmli buffer system (0.75

M Tris in separating gel, 0.05 *M* Tris in reservoir buffer) was used to achieve optimal resolution of low M_r peptides [32]. Electrophoresis was performed at 60 V until the dye front passed through the stacking gel (four hours) and then at 35 mA constant current until electrophoresis was completed. Peptides were electroblotted onto PVDF membrane (as described above) and, after visualization with Coomassie blue R-250, excised and subjected to sequence analysis.

Method 3: In situ proteolysis of proteins in gel slices followed by extraction and separation of peptides by microbore column RP-HPLC. Proteins were separated by one-dimensional SDS-PAGE, visualized by staining with Coomassie blue R-250 as described in Method 2 and the protein-containing gel slices were excised and soaked in water for 24 h. The gel slices were partially dried (*ca.* 50% by weight) in a centrifugal vacuum concentrator (Savant), rehydrated in 100 μl of 100 m*M* NH_4HCO_3, 0.5 m*M* $CaCl_2$, 0.02% (w/v) Tween 20 containing trypsin (enzyme-to-substrate mass ratio was 1:10) and then incubated at 37°C for 12 h. After proteolysis, peptide fragments were extracted from the gel slices with trifluoroacetic acid containing 0.01% Tween 20, essentially as described by Eckerskorn *et al.* [33]. Briefly, the gel slices were extracted for four hours with 100 μl of 1% (v/v) trifluoroacetic acid, for four hours with 100 μl of 70% (v/v) trifluoroacetic acid (twice) and, finally, for four hours with 100 μl of a trifluoroacetic acid–acetonitrile (50:50, v/v) mixture (twice). The combined eluents (*ca.* 500 μl) were concentrated (*ca.* ten-fold) by lyophilization (Savant). This peptide mixture was diluted to 1 ml with 0.1% (v/v) trifluoroacetic acid and the peptides were separated by microbore RP-HPLC [1,23–25] for sequence analysis as described above.

Method 4: Direct elution of proteins from polyacrylamide gels. β-Lactoglobulin (20 μg) was electrophoresed and directly eluted from the polyacrylamide matrix using the Applied Biosystems Model 230A electrophoresis apparatus (see above). Protein-containing fractions were pooled and protein was recovered from the elution buffer (free from SDS, salts and acrylamide-related artefacts) by inverse-gradient RP-HPLC (see Method 1).

Amino acid sequence analysis

Automated Edman degradation of peptides was performed using Applied Biosystems sequencers (Models 470A or 477A) equipped with Model 120A PTH-amino acid analyzers as previously described [25]. Injection of the total amount of PTH-amino acid derivative was facilitated by the installation of an improved sample transfer device described elsewhere [34].

Amino acid analysis

Protein concentration and amino acid composition was determined by amino acid analysis. Samples were hydrolyzed *in vacuo* at 110°C for 24 h with 6 *M* HCl containing 1% (w/v) phenol. Amino acid analyses were performed on a Beckman (Model 6300) analyzer equipped with a Model 7000 data handling system.

RESULTS AND DISCUSSION

Elution of electroblotted proteins from PVDF membrane, enzymatic digestion followed by microbore RP-HPLC separation of peptides (Method 1)

A typical example of this method employing β-lactoglobulin is given in Fig. 1. β-Lactoglobulin (20 μg) was electrotransferred from a one-dimensional polyacrylamid e gel onto PVDF membrane. After visualization by staining with Coomassie blue R-250, β-lactoglobulin was eluted from the membrane with a detergent mixture comprising 2% (w/v) SDS, 1% (w/v) Triton X-100 and 0.1% (w/v) dithiothreitol. We have previously demonstrated [5], using a number of proteins of varying molecular weight, that 57–78% protein recoveries from PDVF membranes can be achieved with this detergent regimen. Before enzymatic digestion it is necessary to separate β-lactoglobulin from the detergent mixture since most commonly used proteases are not active in low concentrations (>0.1%) of SDS. Alternative, non-detergent eluents (*e.g.* organic solvent–trifluoroacetic acid) typically resulted in lower protein recoveries (in the range, 23–42%) [5].

Inverse-gradient RP-HPLC was employed to recover β-lactoglobulin from the

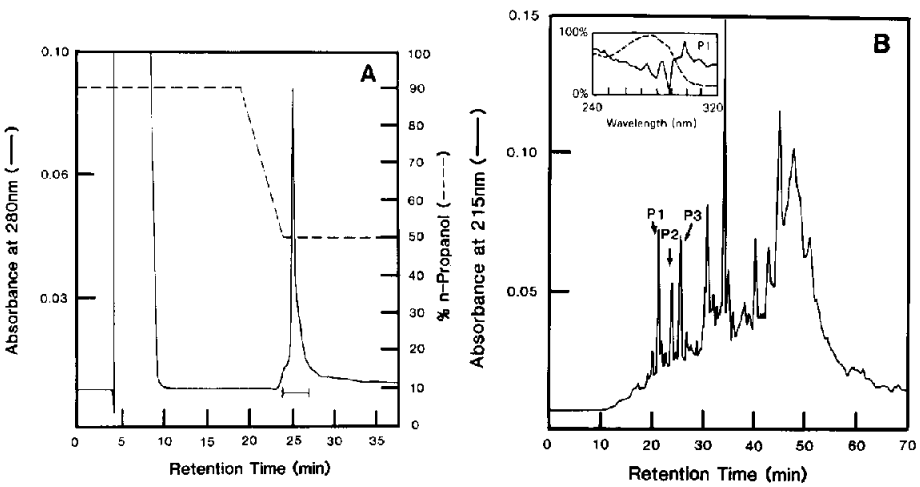

Fig. 1. Peptide mapping of electroblotted β-lactoglobulin by Method 1. β-Lactoglobulin (20 μg) was electrophoresed by SDS-PAGE, electroblotted onto a PVDF membrane, visualized with Coomassie blue R-250 and then eluted from the PVDF membrane with a detergent mixture (see Materials and Methods). β-Lactoglobulin-containing fractions (shown by horizontal bar) were proteolytically digested with pepsin. (A) Recovery of β-lactoglobulin (6 μg) from detergent eluate by inverse-gradient RP-HPLC. Chromatographic conditions: Brownlee VeloSep C_8 cartridge (40 × 3.2 mm I.D.) previously equilibrated with 90% (v/v) aq. 1-propanol; the column was developed at 200 μl/min with a linear 10-min gradient from 100% A to 100% B where solvent A was 90% (v/v) aq. 1-propanol and solvent B was 50% (v/v) aq. 1-propanol containing 0.4% (v/v) trifluoroacetic acid. (B) Separation of peptic peptides of β-lactoglobulin from Fig. 1A by microbore RP-HPLC. Chromatographic conditions: column, Brownlee RP-300 (100 × 2.1 mm I.D.); the column was developed with a linear 60-min gradient from 100% A to 100% B, where eluent A was 0.1% (v/v) aq. trifluoroacetic acid and eluent B was 60% aq. acetonitrile, containing 0.09% (v/v) trifluoroacetic acid. Flow-rate was 100 μl/min. Real-time spectral analysis of peptide P1 (inset), the dotted and solid line corresponding to the first derivative and second derivative absorbance spectra, respectively. The indicated peptides were subjected to amino acid sequence analysis (Table I).

detergent mixture free from Coomassie blue, detergents and gel-related artifacts (Fig. 1A). Previously, we have shown that these contaminants seriously interfere with both peptide mapping by RP-HPLC and the Edman degradation procedure [4]. Material recovered from the inverse-gradient RP-HPLC step was proteolyzed with pepsin and generated peptides were fractionated by RP-HPLC (Fig. 1B). Selected peptides from Fig. 1B were subjected to sequence analysis (see Table I).

Overall peptide yields, determined by N-terminal sequence analysis and expressed as a percentage of the amount of protein loaded on the gel were, typically, 1–4% (Table I). It should be noted that the recovery of protein electroblotted from gel to PVDF membrane was *ca.* 33%, (determined by amino acid analysis), hence, peptide yields from electroblotted β-lactoglobulin, using this approach, were 4–15%.

Photodiode-array detection was employed in the peptide mapping studies to obtain spectral information on purified peptides. A derivative UV absorbance spectrum (240–320 nm) of a representative peptic peptide, P1, is shown as an insert in Fig. 1B. An inspection of the spectrum reveals that it contains aromatic amino acids as evidenced by their absorption peaks in the range 270–290 nm. Enhancement of resolution by second-order derivative spectroscopy reveals a characteristic minimum at 290 ± 2 nm for peptic peptide P1; previously, we [35,36] and others [37] have shown that minima at 290 nm (± 2 nm) and 278 nm (± 2 nm) are characteristic of tryptophan residues whilst a single minimum at 280 nm (± 2 nm) is characteristic of tyrosine residues. The presence of a tryptophan residue in peptic peptide P1 was confirmed by amino acid sequence analysis (Table I).

In situ proteolytic digestion of proteins in a polyacrylamide matrix, separation of the peptides by SDS-PAGE followed by electrotransfer of peptides onto PVDF membranes (Method 2)

In this three-step procedure, the protein is first separated using either one- or two-dimensional polyacrylamide gel electrophoresis, then stained with Coomassie blue R-250. The protein of interest was excised and then digested *in situ* in the polyacrylamide matrix with *Staphylococcus aureus* V8 protease [31]. Resultant peptides were separated, by SDS-PAGE, in a second polyacrylamide gel and then electrotransferred onto PVDF membrane. The electroblotted peptides were visualized by staining with Coomassie blue R-250, excised and directly sequenced. A representative example of this method, using β-lactoglobulin, is given in Fig. 2. Sequence data from selected peptide bands, indicated in Fig. 2, are given in Table I. Despite the very low overall initial sequencing yields obtained (0.1–7.4% of protein loaded onto gel, see Table I), useful sequence information can be obtained. These low yields can be ascribed to: (i) incomplete protein digestion with *S. aureus* V8 protease, where it should be noted that partial digestion is inherent in the Cleveland procedure [31] in order to obtain peptide fragments of a size suitable for separation by SDS-PAGE; (ii) as mentioned above, the large sample losses were incurred during transfer to PVDF membranes. This approach has been successfully employed elsewhere to obtain internal sequence data for a number of proteins including ribosomal proteins [38], Aplysia proteins [39] and receptor proteins [40].

One disadvantage of this approach, the separation of low M_r peptides can be alleviated by using the Tricine-SDS-PAGE system [41] which separates peptides in the range of 1–2 kDa. We have used this system to isolate cyanogen bromide fragments of

TABLE I

SEQUENCE ANALYSIS OF PEPTIDES GENERATED FROM ELECTROPHORETICALLY SEPARATED PROTEINS[a]

Sample	Initial yield in first cycle[b] (pmol)	Overall yield[c] (%)	Sequence matched[d]	Positions of residues sequenced
Elution of electroblotted proteins from PVDF membrane, recovery of protein from detergent by inverse-gradient RP-HPLC, proteolytic digestion, separation of peptides by RP-HPLC (Method 1)				
β-Lactoglobulin[e]				
peptic peptide P1	14	1.3	QKVAGTW	13–19
peptic peptide P2	13	1.2	YVEELKPTPE	42–51
peptic peptide P3	49	4.4	LIVTQTMKGL	1–10
In situ proteolytic digestion of protein, electrophoretic separation of peptides, electroblotting of peptides onto PVDF membrane (Method 2)				
β-Lactoglobulin				
S. aureus V8 peptide S1	32	2.9	LIVTQTMKGLDIQKVAGTXYS	1–21
S. aureus V8 peptide S2	82	7.4	LIVTQTMKGLDIQKVAGTXYSL	1–22
S. aureus V8 peptide S5	9	0.1	ILLQKXEND	26–64
In situ proteolytic digestion, extraction, separation of peptides by RP-HPLC (Method 3)				
β-Lactoglobulin				
tryptic peptide T1	191	17.2	TPEVDDEAL(E)K	125–135
tryptic peptide T2	61	5.5	WENDE(C)AQK	61–69
tryptic peptide T3	460	41.4	ALPMHIR	142–148
tryptic peptide T4	82	7.3	LIVTQTMK	1–8
tryptic peptide T5	25	2.3	LSFNPTQLEEQ(C)HI	149–162
Interleukin-6				
tryptic peptide T1	438	47.6	LPEIQR	66–71
tryptic peptide T2	327	35.5	EWLR	159–162
tryptic peptide T3	210	22.8	SLEEFLK	172–178
tryptic peptide T4	24	2.6	TIQFILK	165–171
tryptic peptide T5	8	0.1	ISSGLLEYHSYLEYMK	89–104
tryptic peptide T6	139	15.1	ISSGLLEYHSYLEYMK	89–104
tryptic peptide T7	608	66.8	DTETLIHIFNQEVK	121–134
tryptic peptide T8	107	11.6	IVLPTPISNALLTDKLESQK	139–158
tryptic peptide T9	5	0.1	FTED	11–14

[a] Total protein loaded onto gel: β-lactoglobulin (20 μg, 1100 pmol), interleukin-6 (20 μg, 921 pmol). The one-letter notation for amino acids has been used. PTH-cysteine was detected as an unidentified adduct (see Fig. 5). Ambiguous amino acid assignments are in parentheses. X denotes unidentified amino acid in that cycle.

[b] Amount of PTH-amino acid (pmol) in the first cycle of Edman degradation.

[c] Overall percentage yield calculated as the ratio of initial yield of PTH-amino acid derivative in the first cycle of the Edman degradation to the total amount of protein loaded onto gel (incorporates yields through electrophoresis, electroblotting and sequence analysis).

[d] The amino acid sequence for β-lactoglobulin (molecular mass, 18.4 kDa) was taken from refs. 58 and 59 and from that of murine interleukin-6 (molecular mass, 21.7 kDa) from ref. 25.

[e] Yield of β-lactoglobulin transferred from gel to PVDF membrane, determined by amino acid analysis, was 6 μg (330 pmol).

proteins digested *in situ* in the polyacrylamide gel [42]. *In situ* enzymatic digestion during electrophoresis as performed here relies on the comigration of enzyme and protein during the stacking process [31]. The differing stacking and destacking limits of

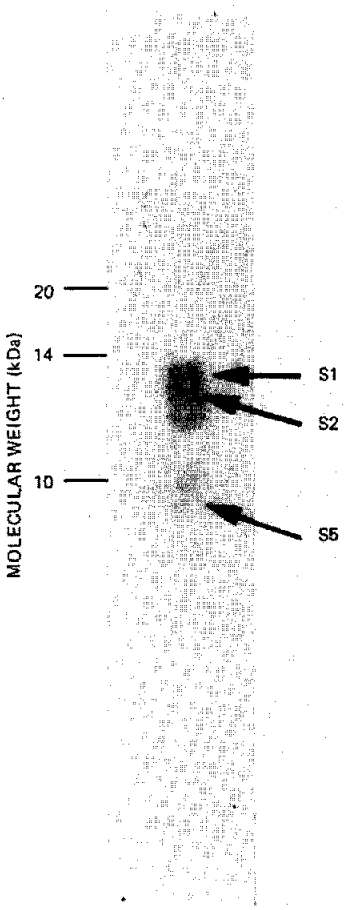

Fig. 2. Peptide mapping of β-lactoglobulin by Method 2. Coomassie blue-stained profile of *S. aureus* protease digest of β-lactoglobulin (20 μg), prepared according to the method of Cleveland [31], after electroblotting onto PVDF membrane. Peptide bands labelled S1, S2 and S5 were excised and subjected to sequence analysis (Table I).

the Tricine system, compared to the Laemmli system, make it unsuitable for this purpose as proteins stack into distinct zones in the Tricine stacking gel and prevent interaction of protease and substrate. Cleavage of the protein must therefore be performed prior to electrophoresis if Tricine gels are to be used for peptide separation.

In situ proteolysis in polyacrylamide matrix followed by extraction and separation of peptides by microbore RP-HPLC (Method 3)

To overcome the high losses during electrotransfer of protein and peptides from gels to PVDF membranes, we decided to examine methods for circumventing this step. Previously, it has been shown that proteins can be enzymatically digested *in situ* in gel slices and resultant peptides extracted under both acidic (trifluoroacetic acid) [33] or

alkaline conditions [50 m*M* N-methylmorpholine acetate (pH 8.1)] [43]. Examples of this approach, using β-lactoglobulin and recombinant murine interleukin-6, are shown in Fig. 3. In these examples, β-lactoglobulin and interleukin-6 were electrophoresed on a one-dimensional SDS-polyacrylamide gel. After visualization by staining with Coomassie blue R-250, the protein bands were cut out and treated *in situ* with trypsin. Resultant peptides were extracted with trifluoroacetic acid and fractionated by microbore column RP-HPLC (Fig. 3A and B).

It can be seen in Table I that the initial sequencing yields of peptides isolated and sequenced by this method, expressed as a percentage of sample loaded, were 2.3–41.4% for β-lactoglobulin and 0.1–66.8% for interleukin-6. These recoveries were significantly higher than those achieved by electrotransfer of proteins or peptides onto PVDF membranes (Methods 1 and 2). It should be noted that higher yields were obtained for PTH-tryptophan in peptide T2 of both β-lactoglobulin and interleukin-6 (Table I) (data not shown). These data suggest that possible chemical damage to amino acids (particularly tryptophan and methionine) during electrophoresis, reported by others [43,44], can be minimized if gels are pre-electrophoresed before sample application [44] and care is taken to avoid overheating during the electrotransfer step (data not shown).

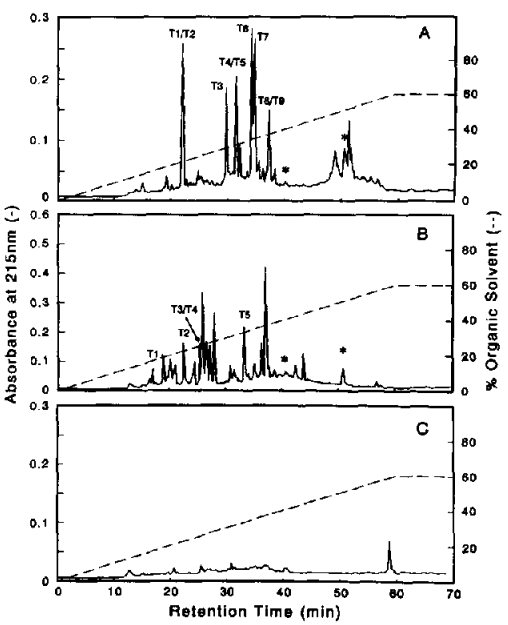

Fig. 3. Peptide mapping of interleukin-6 and β-lactoglobulin by Method 3. After one-dimensional SDS-PAGE, proteins were proteolytically digested *in situ* in the gel with trypsin and the generated peptides extracted from the gel with trifluoroacetic acid and fractionated by RP-HPLC (see Method 3 in Materials and Methods section). Chromatographic conditions were the same as described in the legend for Fig. 1B. (A) Tryptic digest of murine interleukin-6. (B) Tryptic digest of β-lactoglobulin. (C) Enzyme blank (*i.e.*, tryptic digest of gel slice containing no protein). Amount of protein electrophoresed, 20 μg. Artifacts due to Coomassie blue R-250 and gel are indicated by asterisks.

One possible drawback of this method is that Coomassie blue R-250, with its high absorbance at 215 nm, can interfere with the interpretation of HPLC peptide maps. However, if care is taken to minimize the Coomassie blue-to-protein ratio then stain-related artefact peaks can be markedly reduced. The levels of these artefacts can be further reduced by soaking gel pieces in 50% aq. (v/v) 1-propanol containing 3% (w/v) SDS (in a 1.5-ml Eppendorf tube) for 3–4 h at room temperature prior to washing with water (data not shown). This approach has previously been used to remove Coomassie blue from Coomassie blue-stained proteins in gels in order to quantitate separated proteins [45]. The artefact peaks, indicated by asterisks in Fig. 3, panels A and B, were not observed when proteins were digested in solution (Fig. 4A and B). It should be emphasized that Coomassie blue artefact peaks were not observed in Method 1 where the dye was removed during the inverse-gradient RP-HPLC step (see Fig. 1B).

A comparison of the tryptic maps of proteins digested *in situ* in the gel slice (Fig. 3) with that in solution (Fig. 4) reveals comparable patterns, although differences are apparent. Whether these differences reflect conformational differences when the protein is restrained within the gel matrix, reduced accessibility of protein to enzyme or differential extractability of peptides cannot be ascertained from these studies.

Interestingly, sequence analysis of tryptic peptides T2 and T5 of β-lactoglobulin, revealed an unusual PTH-amino acid derivative peak in those cycles of the Edman degradation, which corresponded to cysteine residues. This peak which chromato-

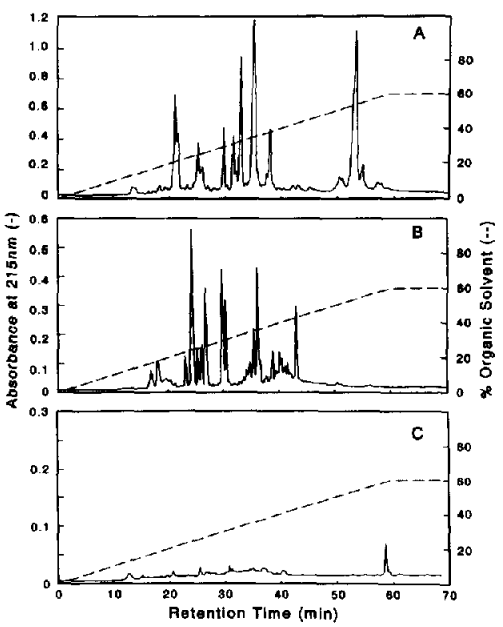

Fig. 4. Peptide mapping of interleukin-6 and β-lactoglobulin by RP-HPLC. Proteins (20 μg) were not subjected to SDS-PAGE, but digested directly with trypsin (2 μg of trypsin in 100 μl of 100 m*M* NH_4HCO_3, 0.5 m*M* $CaCl_2$, 0.02% Tween 20 at 37°C for 12 h). (A) Tryptic digest of interleukin-6; (B) Tryptic digest of β-lactoglobulin; (C) Trypsin blank (*i.e.*, no added substrate). Chromatographic conditions were the same as described in the legend for Fig. 1B.

graphed slightly later than the N',N-dimethyl-N'-phenylthiourea (DMPTU) peak, is illustrated for peptide T2 in Fig. 5. Since these proteins were not reduced and alkylated, derivatization of the cysteine residues during manipulation steps before sequencing seems likely. This derivative may be the same as a recently described cysteine derivative, identified by amino acid analyses, in proteins recovered from polyacrylamide gels by electroblotting [46]. Since cysteine groups are known to be highly reactive with α–β unsaturated compounds such as acrylamide [47], Plough *et al.*, suggest that this derivative may have arisen by reaction of cysteine residues with acrylamide monomer [46].

Peptide fractions obtained from one-dimensional RP-HPLC of enzymatic digest mixtures, although apparently homogeneous as judged by peak shape, often contain more than one peptide upon amino acid sequence analysis. This was the case with peptides T3 and T4 (β-lactoglobulin) and peptides T1/T2, T4/T5 and T8/T9 of IL-6 (Fig. 3) Isolation of pure peptides obviously requires additional chromatographic steps [1,25,36]. The recent advent of capillary electrophoresis [48,49] with its high-resolving power and sensitivity, provides a rapid analytical method for determining the homogeneity of HPLC derived peptides [50]. This approach is illustrated in Fig. 6 where peptide fractions T4/T5 (Fig. 6A), T8/T9 (Fig. 6B) and T6/T7 (Fig. 6C) of

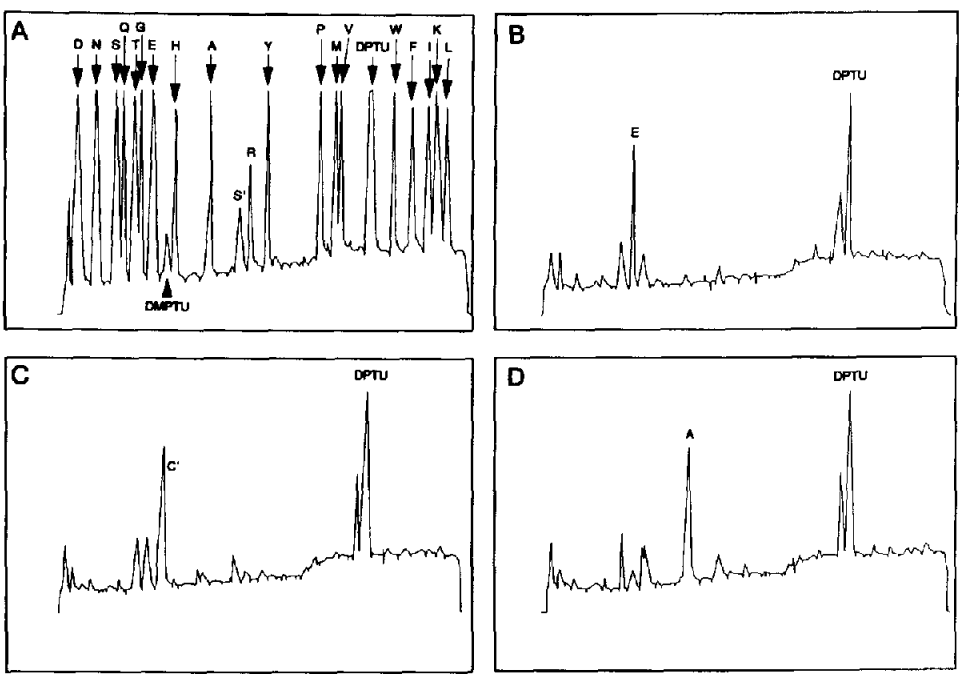

Fig. 5. Sequence analysis of tryptic peptide T2 from β-lactoglobulin (from Fig. 3B). (B–D), PTH-amino acid analyses for Edman degradation cycles 5–7 of tryptic peptide T2 (61 pmol), respectively. The ordinate shows absorbance at 269 nm and the abscissa elution time for each chromatogram. Approximately 95% of the total PTH-amino acid derivative from each cycle was analyzed. A standard PTH-amino acid separation is shown in (A). Amino acids are identified by their single-letter code. DMPTU is N',N-dimethyl-N'-phenylthiourea; DPTU is N',N-diphenylthiourea; C' is an unidentified cysteine derivative.

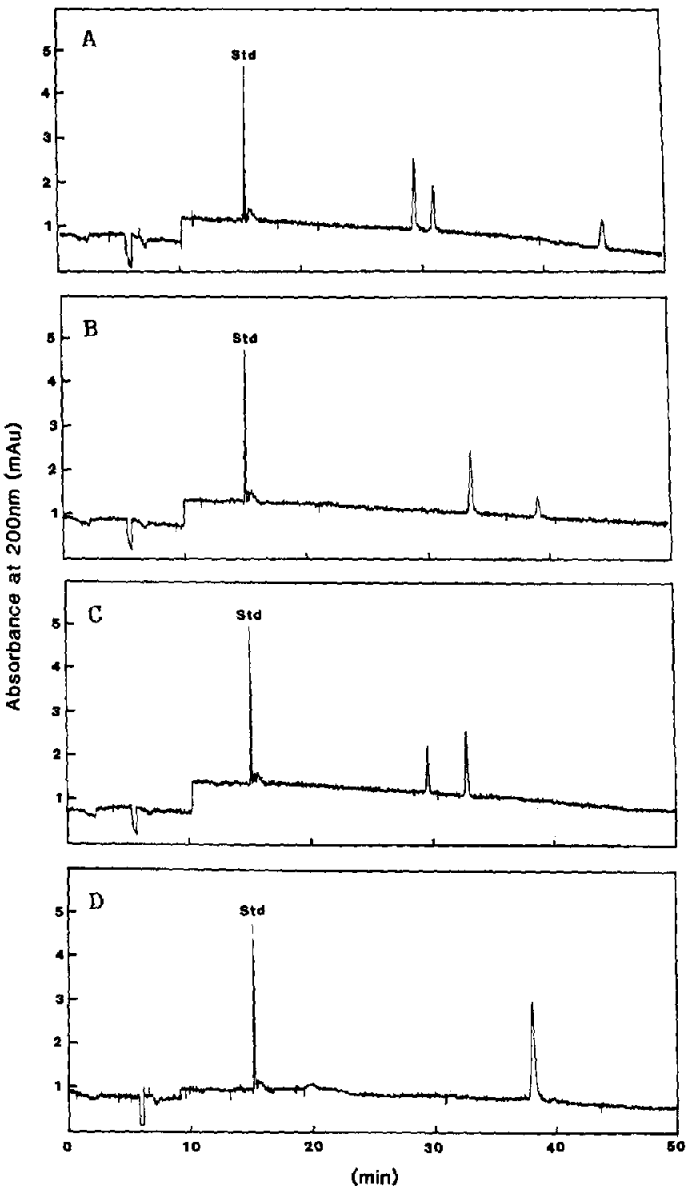

Fig. 6. Electropherograms of peptide fractions from tryptic digests of interleukin-6 and β-lactoglobulin (see Materials and Methods section for details). (A) fraction T4/T5 from Fig. 3A; (B) fraction T8/T9 from Fig. 3A; (C) fraction T6/T7 from Fig. 3A; (D) fraction T5 from Fig. 3B. Capillary: 122 cm × 50 μm I.D., 100 cm to the detector. Capillary was pre-washed with 0.1 *M* NaOH for 5 min prior to injection. Peptide fractions (*ca.* 0.01 mg/ml) in 0.1% (v/v) trifluoroacetic acid in 25–30% acetonitrile were applied to the capillary under vacuum for 5 s. The synthetic peptide, RKRSRKE, was introduced as a mobility marker under vacuum for 3 s. Free solution electrophoresis was performed at 30 kV and 20 μA for 40 min. Buffer: 20 m*M* sodium citrate, pH 2.5.

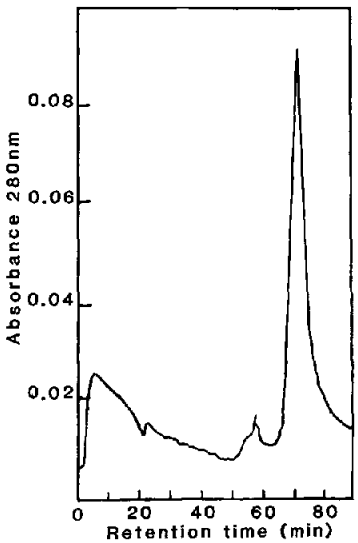

Fig. 7. Direct electrophoretic elution of β-lactoglobulin from an SDS-polyacrylamide tube gel. β-Lactoglobulin (20 μg in 10 μl) was applied onto an 8% acrylamide tube gel (0.25 × 5 cm) and run at 0.8 mA using an Applied Biosystems Model 230A (HPEC) electrophoresis apparatus. Eluate fractions were collected automatically in a fraction collector. Fractions containing β-lactoglobulin were pooled and β-lactoglobulin was recovered from the elute, free from SDS and acrylamide-gel related contaminants, by inverse-gradient RP-HPLC [4,6] (see Materials and Methods Section).

interleukin-6 were resolved under acidic conditions (pH 2.5) into component peaks by capillary electrophoresis. Peptide T5 of β-lactoglobulin was demonstrated to be homogeneous as judged by capillary electrophoresis (Fig. 6D).

Direct elution of proteins from polyacrylamide gels (Method 4)

The direct elution of β-lactoglobulin from an SDS-polyacrylamide disk gel using an Applied Biosystems Model 230A electrophoresis apparatus is shown in Fig. 7. Fractions containing β-lactoglobulin were pooled and the protein concentration determined by amino acid analysis. The recovery of β-lactoglobulin in the pooled fraction was 10 μg, *i.e.*, 50% of the total protein applied to the gel. Since the eluate contained 0.1% (w/v) SDS (as measured by the method of Waite and Wang [51]), buffer salts and acrylamide-related contaminants, it could not be directly applied to the sequencer. β-Lactoglobulin was recovered from the elute, in a form suitable for sequence analysis, by inverse-gradient RP-HPLC. The yield of proteins from this latter step, as assessed by amino acid analysis, was >85%.

CONCLUSIONS

In this study, all strategies investigated for obtaining internal sequence data from electrophoretically separated proteins yielded useful amino acid sequence data. However, compared to *in situ* proteolytic digestion, extraction and separation of peptides by RP-HPLC (Method 3), those strategies which incorporated an electroblotting step

(Methods 1 and 2) resulted in significantly lower initial sequencing yields for isolated peptides. This was due to marked protein losses (60–70%) incurred during the electroblotting step in Method 1 and the additional electrophoresis and electroblotting steps in Method 2.

The problem of sample losses incurred during electroblotting, resulting from poor transfer efficiency, has been studied extensively [52]. From the many reports in the literature concerning procedures for optimizing the efficiency of electrotransfer of proteins onto PVDF membranes (*e.g.* manipulation of buffer salt and methanol concentration in transfer buffer [46], pre-treatment of PVDF with polybrene [52]) it would appear that no one set of transfer conditions is generally applicable for all proteins and that transfer conditions need to be tailored for an individual protein. However, little attention has been given to the potential losses during electrophoresis itself. In this study, only 50% of the protein applied to the acrylamide gel was recovered by direct electrophoretic elution. This finding is in agreement with the studies of Kapadia and Chrambach [53]. Using a direct elution system, these workers showed that irreversible absorption of protein to polyacrylamide gel decreases from 40% to 10% when the protein load was increased from 5 $\mu g/cm^2$ to 3 mg/cm^2 (using a 10% acrylamide gel) [53]. The retention of protein by the acrylamide matrix was also dependent on the percentage of acrylamide used, irreversible absorption increasing from 6%–35% when the acrylamide concentration increased from 6% to 20%. These observations indicate that the 35% recovery of β-lactoglobulin on PVDF membrane in Method 1 cannot be ascribed totally to losses incurred during the electroblotting step. This conclusion is based upon the premise that protein losses incurred by direct elution using the Applied Biosystems Model 230A HPEC system result from irreversible binding to the acrylamide matrix and not from the mode of sample collection. Non-specific binding of protein to the acrylamide gel can be reduced by pre-electrophoresing the gel with protein [53] or including Triton X-100 in the reaction mixture during polymerization [54].

Fragmentation strategies not employing electroblotting steps (*e.g.*, *in situ* proteolytic digestion/extraction, separation of peptides by RP-HPLC and direct electrophoretic elution) have inherent advantages and weaknesses which should be taken into account. For *in situ* proteolytic digestion procedures to work efficiently, as in this study, complete proteolytic digestion is desired since large M_r peptides are not readily extracted from the acrylamide gel with trifluoroacetic acid. This was observed to be a problem with large-M_r cyanogen bromide fragments following *in situ* cyanogen bromide cleavage in polyacrylamide gels [42]. Since most native proteins are resistant to proteolysis, *in situ* reduction and alkylation of protein in the acrylamide gel should facilitate proteolytic digestion. In this regard the pre-electrophoretic alkylation procedure of Gorman [55] may prove useful. In our experience, proteins eluted directly from disk gels (*e.g.*, using the Applied Biosystems HPEC system) are more amenable to further manipulations such as reduction and alkylation. With direct electrophoretic elution from the gel, it should be noted that eluted proteins are collected in buffer volumes directly proportional to their velocity of migration. This is a consequence of the fact that although band width (as viewed by direct staining of the gel) is essentially identical in both cases, the length of the time required to elute all protein from front to rear of the band is much longer for the higher-M_r or slower migrating proteins. For example β-lactoglobulin (18.4 kDa) was collected in *ca.* 0.40 ml while bovine serum

albumin (BSA) (M_r 67 kDa) eluted in *ca.* 0.8 ml. Thus, using direct electrophoretic elution, larger quantities if high-M_r proteins (*ca.* 6 μg) are required for detection at 280 nm as compared to low-M_r proteins (3 μg for β-lactoglobulin).

In situ tryptic digestion of proteins after blotting onto nitrocellulose [56], PVDF or derivatized glass fibre sheets [57] has been used successfully to generate internal amino acid sequence data. A claimed advantage of these methods is that the number of peptides released from the membrane is much smaller than that achieved by extracting peptides from the gel (Method 3) and that this results in simplified HPLC chromatograms [57]. However, in our experience, peptides can be readily resolved from complex peptide maps (*e.g.*, Fig. 3) by multidimensional chromatographic purification steps. This approach is carried out routinely in this laboratory to obtain pure peptides for sequence analysis. For example, the complete amino acid sequence of IL-6 (187 residues) was obtained using 40 μg of protein; 6 μg of IL-6 was used for each individual proteolytic digest and multiple chromatographic steps, employing microbore column RP-HPLC, was used to purify peptides [25]. However, if the level of protein is decreased much below this level (6 μg) the small losses occurring with each chromatographic dimension (10–20%) preclude this approach. With this in mind, the higher yields (as high as 66.9%) and more extensive digests obtained with *in situ* enzymatic digestions in the acrylamide gel matrix (Method 3) far outweigh any disadvantages in the need to perform extra chromatographic dimensions. In this study, capillary zone electrophoresis proved a powerful auxiliary analytical tool in RP-HPLC peptide mapping strategies for assessing peptide purity.

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