Journal of Chromatography, 476 (1989) 345–361
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1628

PEPTIDE MAPPING AND INTERNAL SEQUENCING OF PROTEINS ELECTROBLOTTED FROM TWO-DIMENSIONAL GELS ONTO POLYVINYLIDENE DIFLUORIDE MEMBRANES

A CHROMATOGRAPHIC PROCEDURE FOR SEPARATING PROTEINS FROM DETERGENTS

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SUMMARY

Direct sequence analysis of proteins electroblotted from two-dimensional polyacrylamide gels onto immobilizing matrices provides an efficient technique for obtaining N-terminal sequence data for proteins not amenable to purification by reversed-phase high-performance liquid chromatography (RP-HPLC). We present in this paper a procedure for obtaining peptide fragments from electroblotted proteins for internal amino acid sequence analysis. First, Coomassie Blue-stained proteins are extracted from polydivinylidene difluoride membranes, using a detergent mixture of sodium dodecylsulfate and Triton X-100. Proteins are then separated from the detergent mixture by a chromatographic procedure which relies on the ability of proteins to interact with certain reversed-phase sorbents at high organic solvent concentrations. Under these conditions, detergents and Coomassie Blue are not retained and pass through the column. Proteins are recovered by simultaneously: (i) introducing trifluoroacetic acid into the mobile phase and (ii) decreasing the organic solvent concentration. After proteolytic fragmentation, peptides are purified by microbore-column (1–2 mm I.D.) RP-HPLC for microsequence analysis.

INTRODUCTION

It is now well-recognized that sample preparation is one of the major limitations to obtaining amino acid sequence information from peptides and proteins isolated in subnanomole quantities from their biological source material[1–3]. Although reversed-phase high-performance liquid chromatography (HPLC) is an established tool for purifying a large range of peptides and proteins, it does have limitations with certain classes of proteins (*e.g.*, membrane proteins and large molecular weight, M_r, hydrophobic proteins). For this category of proteins, electrophoretic separation

0021-9673/89/$03.50

provides an important alternative high-resolution technique. Indeed, two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE), pioneered in 1975 by O'Farrell[4], is a high-resolution technique capable of separating thousands of proteins from various biological sources (*e.g.*, tissue extracts and cell lines)[5].

One of the drawbacks in the past with PAGE-purified proteins has been the difficulty in isolating these proteins from the gel in sufficient quantities and in a form suitable for sequence analysis. The most widely used procedures for recovering proteins from sodium dodecylsulfate (SDS)-PAGE include electroelution[6–8], passive elution[9] and electrotransfer (electroblotting) onto an immobilizing matrix[10–20] (see ref. 1 for review). The latter technique has received considerable attention in recent times due to its simplicity (no expensive equipment required), speed, and potential for handling proteins not suited to RP-HPLC technology. Direct N-terminal sequence analysis of proteins electroblotted onto immobilizing matrices [chemically activated glass, polybase-treated glass, polyvinylidene difluoride (PVDF) membrane] from SDS-polyacrylamide gels is now an important strategy for obtaining amino acid sequence information from subnanomole quantities of protein.

Although direct N-terminal analysis of proteins immobilized on PVDF membranes is an important first step in establishing the identity of proteins resolved by gel electrophoresis, for further characterization it is important that immobilized proteins can be efficiently recovered from the PVDF membrane. For instance, protein recovered from the membrane can be used for immunological studies (*e.g.*, raising antisera) as well as high-sensitivity peptide mapping. Internal protein sequence information, obtained by peptide mapping and sequence analysis is of considerable importance for the following reasons: (i) many proteins are N-terminally blocked and are not amenable to the Edman degradation procedure; (ii) the construction of oligonucleotide probes for molecular cloning; (iii) providing confirmatory evidence for cDNA-derived protein sequences; (iv) "fingerprinting" recombinant proteins; (v) complete protein structure determinations; (vi) identifying post-translational modification sites; (vii) epitope mapping; (viii) disulfide bond assignments and; (ix) ligand binding sites.

During sequence analysis, if the N-terminus of a protein is found to be blocked, then useful internal sequence can be obtained by *in situ* cyanogen bromide treatment of the immobilized protein in the sequencer[21]. This approach was recently used to obtain internal sequence for the N-terminally blocked T-cell growth factor P40[22,23]. However, the most widely used procedure for obtaining internal sequence data is by first fragmenting the molecule (either enzymically or chemically), followed by sequence analysis of the RP-HPLC-purified peptide fragments.

In this report we describe a method for obtaining internal sequence information from proteins electroblotted onto PVDF membrane from SDS-polyacrylamide gels. First, Coomassie Blue-stained proteins are passively eluted from the PVDF membrane, employing a mixture of detergents[24]. Eluted proteins are then separated from the Coomassie Blue and detergents by an inverse-gradient RP-HPLC procedure[25,26] which involves retention of the protein on a reversed-phase sorbent at high organic solvent concentrations. The method is based upon earlier reports in the literature[27–29] that at very high organic solvent concentrations ($> 50\%$) proteins can interact strongly with certain silica-based reversed-phase packings. Interestingly, those packings which best exhibit this behaviour (*i.e.*, U-shaped or bimodal dependency) are characterized

by small pore sizes (6–12 nm), large surface areas (200–400 m^2/g), and high carbon content (7–15%). Using such sorbents, SDS, Coomassie Blue, and gel-related artifacts are not retained at high organic solvent concentrations (*e.g.*, 90% aq. propanol or acetonitrile) and pass through the column: proteins, which strongly interact with the packing under these conditions can be recovered in high yield (>90%) by the simultaneous addition of: (i) an ion-pairing agent (*e.g.*, trifluoroacetic) to the mobile phase and (ii) the introduction of a gradient of decreasing organic solvent (*i.e.*, an "inverse gradient"). Proteins recovered from eluates from SDS-PAGE by this means are free of high concentrations of SDS and acrylamide-related contaminants, and suitable for proteolytic digestion. After enzymatic cleavage, peptide fragments are separated by microbore column (1–2 mm I.D.) HPLC[1–3,30] and subjected to sequence analysis. The general utility of this technique is illustrated for a number of proteins, isolated by either one-dimensional (SDS-PAGE) or by 2D gel electrophoresis.

EXPERIMENTAL

HPLC apparatus

The chromatographic equipment consisted of a Hewlett-Packard (Waldbronn, F.R.G.) liquid chromatograph (HP 1090A), equipped with an autosampler and diode-array detector (HP 1040A). Spectral and chromatographic data were stored on disc, using a Hewlett-Packard Model 85B computer and a Model 9153B disc drive. Spectral analysis (*e.g.*, derivative spectroscopy) were performed with the EVALU 1 and 2 software packages obtained from Hewlett-Packard. 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 were used in this study: (a) Brownlee VeloSep [either, 3 μm octylsilica (C_8) or octadecylsilica (C_{18}), 10 nm pore size, packed into a 40 × 3.2 mm I.D. cartridge], obtained from Applied Biosystems (Foster City, CA, U.S.A.). (b) Hypersil C_{18} (5 μm octadecylsilica, 12 nm pore size, packed into either a 20 × 2.1 mm I.D. cartridge or 100 × 2.1 mm I.D. column or 3 μm octadecylsilica, packed into a 60 × 4.6 mm I.D. column), obtained from Hewlett-Packard. Hypersil C_{18} microbore columns (50 × 2.1 mm I.D. or 50 × 1.0 mm I.D.) containing 3 μm octadecylsilica (12 nm pore size) were packed as previously described[25]. (c) Brownlee RP-300 C_8 (7 μm dimethyloctyl silica, 30 nm pore size, packed into a 100 × 2.1 mm I.D. cartridge), obtained from Applied Biosystems.

Chemicals and reagents

Cytochrome *c*, bovine serum albumin, α-lactalbumin, ribonuclease, insulin, ovalbumin, α-amylase, carbonic anhydrase, transferrin, trypsin inhibitor, 3-(cyclohexylamino)-1-propane sulfonic acid (CAPS), thioglycolic acid, tris(hydroxymethyl)-aminomethane (Tris) base and 2-mercaptoethanol were purchased from Sigma (St. Louis, MO, U.S.A.). SDS was obtained from British Drug Houses (Poole, U.K.). Chymotrypsin and pepsin were purchased from Worthington (NJ, U.S.A.). Proteins were ^{125}I radioiodinated by the chloramine-T procedure[31]. Iodoacetic acid (puriss. grade) from Fluka (Buchs, Switzerland) was recrystallized prior to use. Dithiothreitol

was obtained from Calbiochem (San Diego, CA, U.S.A.). HPLC-grade organic solvents were purchased from Mallinckrodt (Melbourne, Australia). Trifluoroacetic acid (99 + % grade), Tween 20, Brij-35, Triton X-100, and Lubrol-PX were obtained from Pierce (Rockford, IL, U.S.A.). Deionised water, obtained from a tandem Milli-RO and Milli-Q system (Millipore, Bedford, MA, U.S.A.) was used for all buffers. Nonidet P40, Coomassie Blue R250 and ampholines (pH 3.5–10) were purchased from LKB (Bromma, Sweden). PVDF membrane (Immobilon) was purchased from Millipore. Styles of defined S-genotype from the tobacco plant *Nicotinia alata* were obtained as described previously[32].

Preparation of Nicotiana alata style extracts

Protein extracts were prepared from 40 styles from various homozygous clones of *N. alata*, using procedures described elsewhere[32].

Electrophoretic techniques

Style extract proteins were fractionated by 2D gel electrophoresis, using the non-equilibrium pH gradient (NEPHGE) procedure[33]. First-dimension isoelectric focusing gels [4% acrylamide–8.0 *M* urea–2% ampholytes (pH 3.5–10)–2% non-ionic detergent NP-40] were polymerized for 1.5 h. Typically, 100 μg protein was applied to the NEPHGE gels, and electrophoresis was performed at 500 V for 4 h. After NEPHGE, the gels were extruded into 5 ml of SDS-equilibration buffer[4] and stored at −20°C. SDS-PAGE gels (12.5% acrylamide, 1.5 mm thick and 16 cm in length) for the second dimension were prepared the previous day and pre-electrophoresed for 16 h (60 V, 80 mA) with running buffer [0.38 *M* Tris–HCl buffer (pH 8.8), containing 0.1 m*M* thioglycolic acid)]. Equilibrated NEPHGE gels were positioned on top of the second-dimension SDS-PAGE gels using 1.0% (w/v) aqueous agarose. Electrophoresis (240 V, 25 mA) was performed, using the Laemmli SDS-running buffer[34] until the dye-front (bromophenol blue) had migrated 15 cm into the resolving gel.

Electroblotting

After 2D gel electrophoresis, gels were equilibrated for 15 min in transfer buffer (10 m*M* CAPS–10% methanol (pH 11.0)[13]. Prior to use, PVDF membranes were rinsed with 100% methanol, soaked in water for 5 min, and then stored in transfer buffer for at least 15 min. Electrotransfer of proteins from 2D gels onto PVDF membranes was conducted at 4°C in a standard blotting apparatus, the Bio-Rad "Trans-Blot" cell, employing a Model 250/2.5 constant voltage power supply. Electrophoretic conditions: 90 V (300 mA) for 2 h.

Visualisation of blotted proteins

Immediately after electroblotting, blotted proteins were detected by soaking the PVDF membrane in methanol–acetic acid–water (50:10:40, v/v/v), containing 2% (w/v) Coomassie Blue R250, for 10 min. Destaining was performed by soaking the membrane in methanol–acetic acid–water (50:10:40, v/v/v) for 10 min, following by rinsing in water for 10 min. The PVDF sheets were air-dried for 20 min and then stored at −20°C. Alternatively, selected spots for sequence analysis were cut out as disks and stored at −20°C under nitrogen in polypropylene (Eppendorf) tubes.

Amino acid sequence analysis

Automated Edman degradation of electroblotted proteins was performed in an Applied Biosystems sequencer (Model 470A), equipped with an on-line phenylthiohydantoin (PTH) amino acid analyzer (Model 120A) with an improved sample transfer device[35]. Typically, PVDF strips containing the protein were partially sliced and positioned upon a Polybrene[36]-treated glass filter in such a way as to achieve optimal reagent/solvent flow.

Amino acid analysis

Amino acid analysis was performed on a Beckman amino acid analyser (Model 6300), equipped with a Model 7000 integrator. Samples were hydrolysed, *in vacuo*, in 6 *M* HCl containing 0.1% (w/v) phenol for 18 h at 110°C.

Elution of proteins from PVDF

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]. Briefly, the PVDF strip containing the blotted protein was excised, placed in a polypropylene (Eppendorf) tube and soaked with 100 μl detergent mixture at 25°C for 30 min. The eluent was removed by centrifugation at 20 000 *g*, the procedure was repeated, and the eluates were combined.

Preparation of S-carboxymethyl (CM) S_7-glycoprotein

Dithiothreitol was added to the combined S_7-glycoprotein eluents (*ca.* 15 μg in 200 μl) to achieve a final concentration of 10 m*M*, and the mixture was heated for 5 min at 95°C. After reduction, the S_7-glycoprotein was alkylated by the addition of iodoacetic acid (final concentration, 50 m*M*) for 30 min at 25°C in the absence of light. The reaction was halted by the addition of 5 μl of 2-mercaptoethanol.

Chromatographic recovery of proteins from detergent eluent

PVDF-eluted proteins were recovered from the detergent mixture by a chromatographic procedure previously described[25], with minor modifications. Briefly, the aqueous detergent eluate (200 μl) containing *ca.* 6–15 μg of Coomassie Blue-stained protein was diluted in the sample-loading syringe to 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% 1-propanol. The column was developed at 200 μl/min with a linear 10-min gradient from 0 to 100% B where eluent A was 90% aq. 1-propanol and eluent B was 50% aq. 1-propanol, containing 0.4% (v/v) trifluoroacetic acid. The column temperature was 40°C. Peaks were collected manually after correction for post-detector dead volume.

Peptide mapping

Prior to proteolytic digestion, protein samples [*ca.* 6–15 μg in 300 μl 70% aq. 1-propanol 0.2% (v/v) trifluoroacetic acid] were adjusted to 0.02% (w/v) with respect to Tween 20, concentrated three-fold in a centrifugal vacuum concentrator (Savant, Hicksville, NY, U.S.A.) and then diluted to 1 ml with 1% (w/v) ammonium bicarbonate, (pH 7.8), containing 0.02% (w/v) Tween 20 (for chymotrypsin digestion); or 5% formic acid, containing 0.02% (w/v) Tween 20 (for pepsin digestion). Proteins

were digested at an enzyme/substrate mass ratio of 1:20 at 37°C for either 1 h (pepsin) or 4 h (chymotrypsin). Resultant peptide mixtures were fractionated by microbore RP-HPLC, using procedures previously described[1,37].

RESULTS AND DISCUSSION

Two-dimensional gel electrophoresis of Nicotinia alata style extract proteins

Fig. 1 shows a 2D gel of proteins extracted from the styles of a homozygous clone (S_6S_6) of the tobacco plant (*N. alata*). Since the S-allele self-incompatibility glycoproteins located in the style are highly basic[38], NEPHGE was employed in the first electrophoretic dimension to achieve optimal resolution, followed by size fractionation (SDS-PAGE) in the second dimension. Visualization of the proteins on the 2D gel by silver-staining revealed at least 100 clearly resolved spots (Fig. 1). For the purpose of sequence analysis, proteins from identical gels were electrotransferred onto PVDF membranes and visualised with Coomassie Blue R250 (see Experimental section). The major self-incompatibility S-allele glycoproteins from three homozygous clones, (S_2S_2, S_3S_3, and S_6S_6) were chosen for sequence analysis (Table I) along with a number of minor protein spots (P1–P5 from Fig. 1), standards which were selected for the purpose of comparing gels run on different days. In total, 8 proteins were subjected to Edman degradation (Table I) for 15–25 cycles, yielding a total of 82

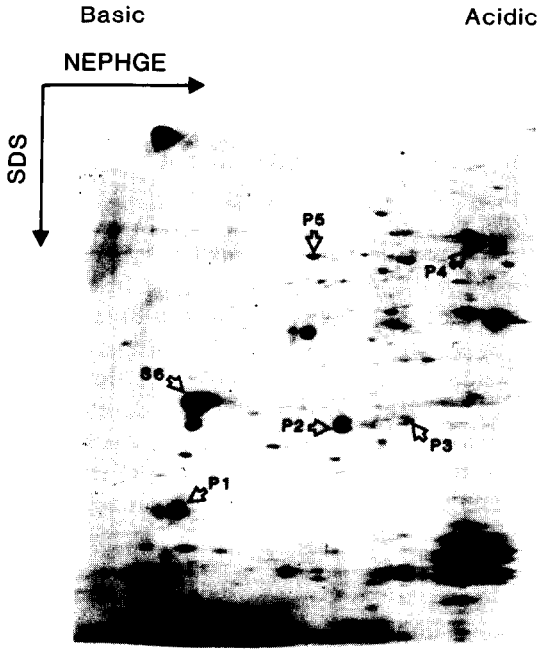

Fig. 1. Two-dimensional gel electrophoresis of *N. alata* style proteins. Total protein extract of 40 styles (*ca.* 100 μg) from homozygous S_6S_6 clones were resolved by non-equilibrium pH gel electrophoresis (horizontal direction) and SDS-PAGE (vertical direction). Proteins were detected by silver-staining. For sequence analysis, proteins from identical gels were electroblotted onto PVDF membranes and then visualised with Coomassie Blue R250. The numbered proteins indicate those selected for sequence analysis (Table I).

TABLE I

N-TERMINAL SEQUENCES OF *NICOTINIA ALATA* STYLE PROTEINS, RESOLVED ON 2D GELS AND ELECTROBLOTTED ONTO PVDF MEMBRANES

Proteins are denoted as shown in Fig. 1 with the exception of S_2 and S_3, which were obtained from analogous 2D gels (not shown). The amino acid sequence is given in the one-letter notation. ?, positions where an unambiguous PTH amino acid assignment could not be made; X, a modified PTH amino acid residue in P1 with a retention time previously not seen in our PTH-amino acid analysis system. Blocked proteins did not yield any amino acid sequence.

Protein	$M_r \cdot 10^{-3}$	*Sequence*																			
		1				5					10					15					20
S_2	31	A	F	E	Y	M	Q	L	V	L	T	W	P	I	T	F	?	R	I	K	
S_3	33	A	F	E	Y	M	Q	L	V	L	Q	W	P	A	A	F	?	H	T	T	P
S_6	32	A	F	E	Y	M	Q	L	V	L	Q	W	P	T	A	F	?	H	X		
			X																		
P1	25	A	P	I	S	K	P	N	?	N	?	N	A	?	Q						
P2	30	?	F	D	?	L	?	L	V	L	T	?	P								
P3	31	D	L	P	V	L	S	E	V	L											
P4	57	Blocked																			
P5	54	Blocked																			

residues. The sequencing efficiencies of this procedure are summarized in Table II. The average repetitive sequencing yields for these proteins, 94–95%, were obtained with an initial coupling yield in the first cycle of 15–22 pmol.

In this study, care was taken to minimize possible chemical modification of proteins (*e.g.* N-terminal blockage and oxidation of tryptophan and methionine residues) during electrophoresis by: (i) introducing an antioxidant (*e.g.*, 0.1 m*M* thioglycollate) in the electrophoresis buffer and (ii) conducting pre-electrophoresis (approximately 12 h) of the gel prior to sample loading. Previously, we[1] and others[11,14,19] had shown that significant improvements in the overall sequencing yields can be obtained by these measures. For instance, the levels for PTH-methionine and -tryptophan in proteins S_2, S_3, and S_6 (residues 5 and 11, respectively) indicate no significant destruction of these labile amino acids (data not shown). If this

TABLE II

REPETITIVE YIELDS (RY) FOR S-GLYCOPROTEINS FROM *NICOTINIA ALATA*

Repetitive yields were calculated using Residues Leu-7 and Ile-13 (S_2). Quantitation of PTH amino acids in each Edman degradation cycle was determined by measuring the peak areas of the PTH signals in each cycle and in a 12.5-pmol standard mixture. Initial yield is the extrapolated value (pmol) of PTH amino acid in cycle 1, determined from a least-squares plot of the data from the sequence run. S_3 was electrophoresed under the neutral conditions described by Moos *et al.*[14].

Protein	$M_r \cdot 10^{-3}$	*Initial yield (pmole)*	*No. of residues identified*	*RY (%)*
S_2	31	22	18/19	94.0
S_6	32	12	16/17	94.7
S_3	33	98	19/20	95.8

TABLE III

ELUTION OF ELECTROBLOTTED PROTEINS FROM PVDF MEMBRANES BY VARIOUS ELUENTS

Proteins were radiolabelled with ^{125}I, electrophoresed on SDS-PAGE, and electrotransferred to PVDF membranes (*cf.* Experimental). After visualization with Coomassie Blue R250, protein spots were excised, placed in an Eppendorf tube and passively eluted with 2 × 100 μl eluent (30 min, 25°C). All detergent elutions were carried out with 50 m*M* Tris–HCl buffer (pH 9.0). Amount of protein loaded onto SDS-PAGE: S_6-glycoprotein, 4 μg; all other proteins, 2 μg. Protein recoveries, determined by measurement of radioactivity, are the amounts of protein eluted, expressed as a percentage of total protein electroblotted onto the PVDF membrane. Values are the average of two experiments performed in triplicate.

Eluent	*Bovine serum albumin, recovery (%)*	*S_6-glyco protein*	*Ovalbumin*	*Carbonic anhydrase*	*Trypsin inhibitor*
2% SDS–1% Triton X-100	64.6	46.0	69.2	74.5	61.4
2% SDS–1% Triton X-100–0.1% DTT	73.1	56.8	74.1	78.0	61.0
2% SDS–1% Nonidet P40	64.7	45.1	67.7	77.3	60.9
2% SDS–1% Nonidet P40–0.1% DTT	66.3	69.0	70.1	70.1	58.7
2% SDS–1% Tween 20	60.9	46.8	59.7	71.3	59.0
2% SDS–1% Tween 20–0.1% DTT	61.6	43.6	60.6	72.6	56.4
2% SDS–1% Lubrol	55.7	27.8	57.6	72.8	55.9
2% SDS–1% Brij-35	56.6	29.7	56.0	73.4	57.2
70% 1-propanol–5% TFA	44.0	41.9	23.2	45.1	38.3
70% 1-propanol–5% TFA–0.1% DTT	47.1	32.5	29.7	37.8	38.5
20% acetonitrile–2% SDS	15.3	5.1	12.5	11.8	29.3

pre-electrophoresis step with an antioxidizing agent is omitted, significant destruction of tryptophan in PVDF-immobilized proteins can result[13].

In contrast to other reports[12], we do not find abnormally low yields of PTH-Asp and PTH-Glu when sequencing PVDF-immobilized proteins (data not shown). It should be noted that for optimal sequencer performance we routinely position the PVDF-immobilized protein strips on top of a Polybrene (3 mg)-conditioned filter disc[1]. This is particularly important when handling peptides or very hydrophilic proteins[1].

Elution of electroblotted proteins from PVDF membranes

We have examined a number of different regimens for eluting proteins from PVDF after their transfer from SDS-polyacrylamide gels. Inspection of Table III reveals that electroblotted proteins of varying molecular weight can be efficiently eluted from PVDF membranes by various regimens of detergents and, to a lesser extent, mixtures of organic solvent and trifluoroacetic acid.

For high-molecular-weight proteins, a small but significant increase in yield was obtained with the addition of 0.1% dithiothreitol (DTT) to the eluent (2% SDS–1% Triton X-100).

HPLC procedure for recovering proteins from detergent eluates

In our earlier studies[25] with inverse-gradient HPLC we employed ODS-Hypersil (3 μm or 5 μm, 12 nm pore size), a sorbent commonly utilized for the RP-HPLC of low-M_r substances (*e.g.*, peptides). We have recently extended these studies to examine

TABLE IV

DATA FOR SPHERICAL POROUS SILICA PACKINGS

Data obtained directly from the manufacturers.

Support	*Particle size (μm)*	*Pore size (nm)*	*Surface area (m^2/g)*	*Surface coverage (μmol/m^2)*	*Pore volume (ml/g)*	*Carbon content (%)*
Hypersil C_{18}	3–5	12	170	2.06	0.7	9.5–10.0
Brownlee VeloSep C_8	3	10	200	2.2	0.8	7.4–8
Brownlee VeloSep C_{18}	3	10	200	1.9	0.8	12–13.2
Brownlee RP-300	7	30	80–110	8.7	0.5–0.6	7

the applicability of other commercially available silica-based reversed-phase sorbents for this purpose; details of these packings are given in Table IV.

Of the four sorbents examined in this study, the small-pore-size, large-surface-area sorbents (*e.g.* ODS-Hypersil and Brownlee C_8 VeloSep) exhibited comparable efficiencies for the panel of proteins chromatographed in the inverse-gradient mode; the large-pore-size (30 nm) packing (Brownlee RP-300) was not considered useful in the inverse-gradient mode, since proteins exhibited very large peak bandwidths and, consequently, were recovered in unacceptably large volumes (600–1500 μl) (Fig. 2). As previously reported[25], the ion-pairing agent trifluoroacetic acid modulates protein retention behaviour in the inverse-gradient chromatographic mode, as well as chromatographic efficiencies and protein recoveries. For practical purposes, we routinely use 0.4% (v/v) trifluoroacetic acid in the mobile phase in order to minimize peak bandwidth; under these conditions, proteins were typically recovered in 100–300 μl when using 2.1 or 3.2 mm I.D. columns.

The effect of protein load on peak width for a representative small-pore-size packing (ODS-Hypersil), operated in reversed-phase or inverse-gradient elution

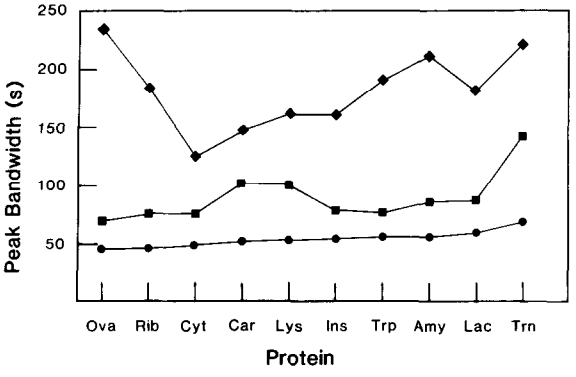

Fig. 2. Plot of the peakwidth (seconds) of eluted proteins from various columns, operated in the inverse-gradient elution mode. Chromatographic conditions are given in Table V. Columns: (◆) Brownlee RP-300 (100 × 2.1 mm I.D.) (●) Brownlee VeloSep C_8 (40 × 3.2 mm I.D.); (■) ODS-Hypersil (100 × 2.1 mm I.D.). Proteins: Ova, ovalbumin; Rib, ribonuclease; Cyt, cytochrome *c*; Car, carbonic anhydrase; Lys, lysozyme; Ins, insulin; Trp, trypsin inhibitor; Amy, α-amylase; Lac, α-lactalbumin; Trn, transferrin.

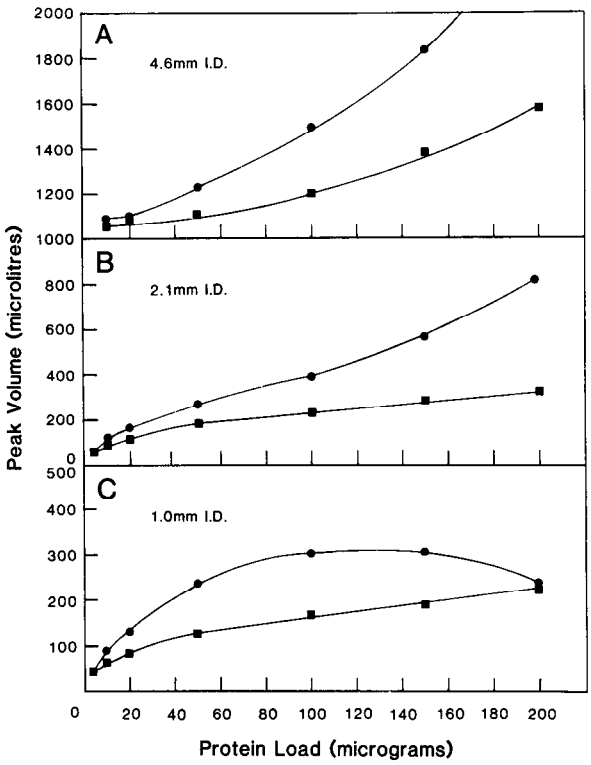

Fig. 3. Effect of protein load on peakwidth of eluted protein for columns of varying internal diameter. Column: ODS-Hypersil (3 μm particle size, octadecyl silica) of varying column dimensions: (A) 60 × 4.6 mm I.D.; (B) 50 × 2.1 mm I.D.; (C) 50 × 1.0 mm I.D. Values are the average of two experiments. Chromatographic conditions: reversed-phase elution mode; linear 50-min gradient from 0–100% B where eluent A is 0.1% (v/v) aq. trifluoroacetic acid and eluent B is 50% aq. 1-propanol, containing 0.1% trifluoroacetic acid. Inverse-gradient elution mode: linear 50-min gradient from 0–100% B, where eluent A is 100% 1-propanol and eluent B is 50% aq. 1-propanol, containing 0.4% (v/v) trifluoroacetic acid. Column temperature 40°C, detection, 280 nm, flow-rates, 1 ml/min, 100 μl/min, and 40 μl/min for 4.6 mm I.D., 2.1 mm I.D., and 1.0 mm I.D. columns, respectively. Sample, α-lactalbumin (varying concentrations in 20 μl water). ●, Inverse-gradient elution mode; ■, reversed-phase elution mode.

mode, is shown in Fig. 3. For sample loads of 1–10 μg, proteins are typically recovered in peak volumes of 70–130 μl from 1–2 mm I.D. columns operated in the inverse-gradient elution mode. These volumes are acceptable for subnanomole structural analysis (*e.g.*, sequence determination in gas-phase/pulsed-liquid sequencers or peptide mapping strategies) where minimization of protein volumes is an important consideration[1]. It is now well established that attempts to concentrate solutions containing subnanomole quantities of protein by classical procedures (*e.g.*, lyophilization or organic solvent precipitation) can result in severe sample loss.

Thus, proteins recovered from detergent mixtures by this means are in an acceptable form for further structural analysis (*e.g.*, mass spectrometry, NMR and peptide mapping). The chromatographic efficiency of proteins in the inverse-gradient mode, *ca.* 90% of that achieved in the reversed-phase mode, is sufficient to facilitate

TABLE V

EFFECT OF PROTEIN LOAD ON PEAK BANDWIDTH

Column: Brownlee C_8 VeloSep (40 × 3.2 mm I.D.). Chromatographic conditions. Reversed-phase elution mode: column was developed with a linear 50-min gradient from 0–100% B where eluent A is 0.1% (v/v) aq. trifluoroacetic acid and eluent B is 50% aq. 1-propanol, containing 0.1% (v/v) trifluoroacetic acid. Inverse-gradient elution mode: column was developed with a linear 50-min gradient from 0–100% B where eluent A is 100% 1-propanol and eluent B is 50% aq. 1-propanol, containing 0.4% (v/v) trifluoroacetic acid. Flow-rate, 400 µl/min, detection, UV at 280 nm, column temperature, 40°C. Peak volumes were determined by measurement of peak width at the peak base. Sample: cytochrome *c* in 20 µl water. Values are peak volume in µl, the values in parenthesis were for samples loaded in the presence of 0.5% SDS.

Amount of protein (µg)	*Chromatographic mode*	
	Reversed-phase	*Inverse-gradient*
0.5	72 (182)	86 (106)
1.0	100 (204)	84 (106)
2.0	97 (224)	93 (112)
4.0	104 (230)	106 (129)
6.0	122 (262)	114 (150)
10.0	111 (297)	129 (165)
15.0	136 (333)	146 (182)
20.0	154 (391)	164 (212)
40.0	200 (450)	218 (288)

their separation (Table V). However, it should be noted that a significant decrease in efficiency (reflected by increased peak volumes) occurs when SDS-containing samples are chromatographed in the reversed-phase elution mode (Table V). This is in marked contrast to chromatography in the inverse-gradient elution mode, where the presence of SDS in the sample does not significantly influence the peak volume of eluted proteins. Thus, the inverse-gradient elution procedure described here offers the potential for resolving proteins at high organic solvent concentrations from detergent mixtures.

Peptide mapping of PVDF-eluted proteins

As mentioned earlier, Coomassie Blue-stained proteins can be efficiently eluted from PVDF membranes with 2% SDS–1% Triton X-100–0.1% dithiothreitol. However, for proteolytic digestion of detergent-eluted proteins, it is necessary to reduce the concentration of detergent, since most proteases used in peptide mapping strategies are inactive in the presence of detergents above concentrations of 0.1–0.2%. Also, it is often necessary to buffer-exchange the eluate to achieve the desired pH (and in some cases, buffer ion) conditions for proteolysis. Although proteins can be separated from detergents by organic solvent precipitation[8,39,40] or by the recently reported[41] size-exclusion chromatography on Sephacryl S-200 with 80% aq. formic acid–methanol (3:1), there are drawbacks with these procedures, the most notable being recoveries, and in the latter case, cleavage of acid-labile peptide bonds (particularly aspartyl–prolyl bonds). In our experience, attempts to achieve acceptable detergent levels (*e.g.*, 0.1–0.2% SDS) for peptide mapping by diluting the eluate were unacceptable, since Coomassie Blue and the ultraviolet-absorbing detergents (*e.g.*,

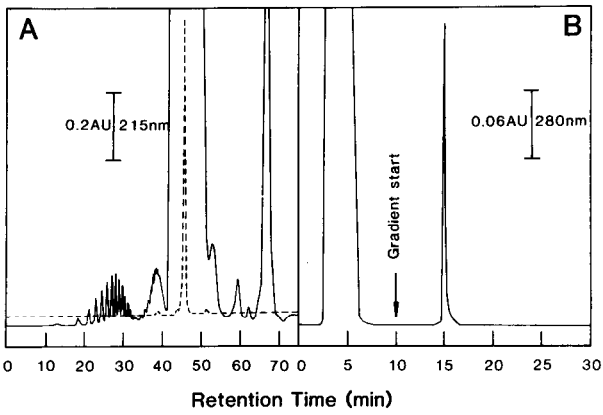

Fig. 4. Chromatography of protein–Triton X-100 mixtures in inverse-gradient and reversed-phase elution modes. Chromatographic conditions: column, Brownlee C_8 VeloSep, 5 μm particle size, 40 × 3.2 mm I.D. (A) Reversed-phase elution mode. The elution position of S_7, determined in the absence of detergent is denoted by the broken line (---); (B) inverse-gradient elution mode (see Table IV for chromatographic details). Sample: 200 μl of aq. 1% Triton X-100, containing 10 μg of S_7.

Triton X-100) were enriched on the reversed-phase column and subsequently interfered with interpretation of the peptide map (Fig. 4). Our approach to this problem is to elute the Coomassie Blue-stained proteins from the PVDF membrane (see Experimental) and to recover the protein from the eluate (free of Coomassie Blue and detergents) by the inverse-gradient HPLC procedure mentioned above (Fig. 4).

As reported earlier[25], SDS concentrations up to 1.2% do not influence the load capacity of small-pore packings, used in the inverse-gradient mode. Beyond this SDS concentration, the amount of protein retained on the column during loading diminishes; this may be due to the reversed-phase packing becoming a dynamic ion exchanger[42]. For example, only 50–60% of a sample protein is retained on a column loaded in the presence of 5% SDS. For samples containing SDS in concentrations greater than 1.2% this problem is circumvented by diluting the sample (in the sample-loading syringe) with up to 1.5 ml of 1-propanol (to reduce the overall SDS concentration) and loaded directly onto the column.

Proteins recovered from detergent eluates by inverse-gradient HPLC (Fig. 5) are then subjected to proteolytic digestion in the polypropylene (Eppendorf) collecting tube. Prior to digestion, the sample is diluted with an appropriate buffer (see Experimental) in order to affect buffer-exchange and to lower the organic solvent concentration (for most proteases, typically, to less than 15%). The resultant peptides were purified by microbore-column (1–2 mm I.D.) RP-HPLC, at a low pH [trifluoroacetic acid (pH 2.1)–acetonitrile system] (Fig. 5). As we have reported elsewhere[1–26,30–34,37], in many cases a second and sometimes a third chromatographic step was required to obtain homogenous peptides. In this application, short columns (< 10 cm) are preferred, since they permit the use of high flow-rates (typically, 0.4–1 ml/min for a 1 mm I.D. column and 0.5–2 ml/min for a 2.1 mm I.D. column). The major advantages of high flow-rates are: (i) rapid trace enrichment of sample onto an interactive sorbent and (ii) rapid column re-equilibration. Previously

we have shown[1-3,37] that short microbore columns do not seriously compromise the chromatographic separation of peptides and proteins on reversed-phase columns.

A summary of sequence data, obtained for selected peptides in this study, is given in Table VI. Fig. 6 shows the HPLC analysis of PTH amino acid derivatives from the Edman degradation of peptic peptide T1, derived from electroblotted β-lactoglobulin (Fig. 5A and B). The good yield of PTH-tryptophan in cycle 7 illustrates that no significant destruction of tryptophan occurs during the electrophoresis/electroblotting/peptide mapping procedure.

It is noteworthy that real-time spectral analysis of eluted peptides was a useful adjunct to this peptide mapping strategy. For instance, the use of derivative ultraviolet absorbance spectra[43,44] allowed the rapid identification of peptides containing

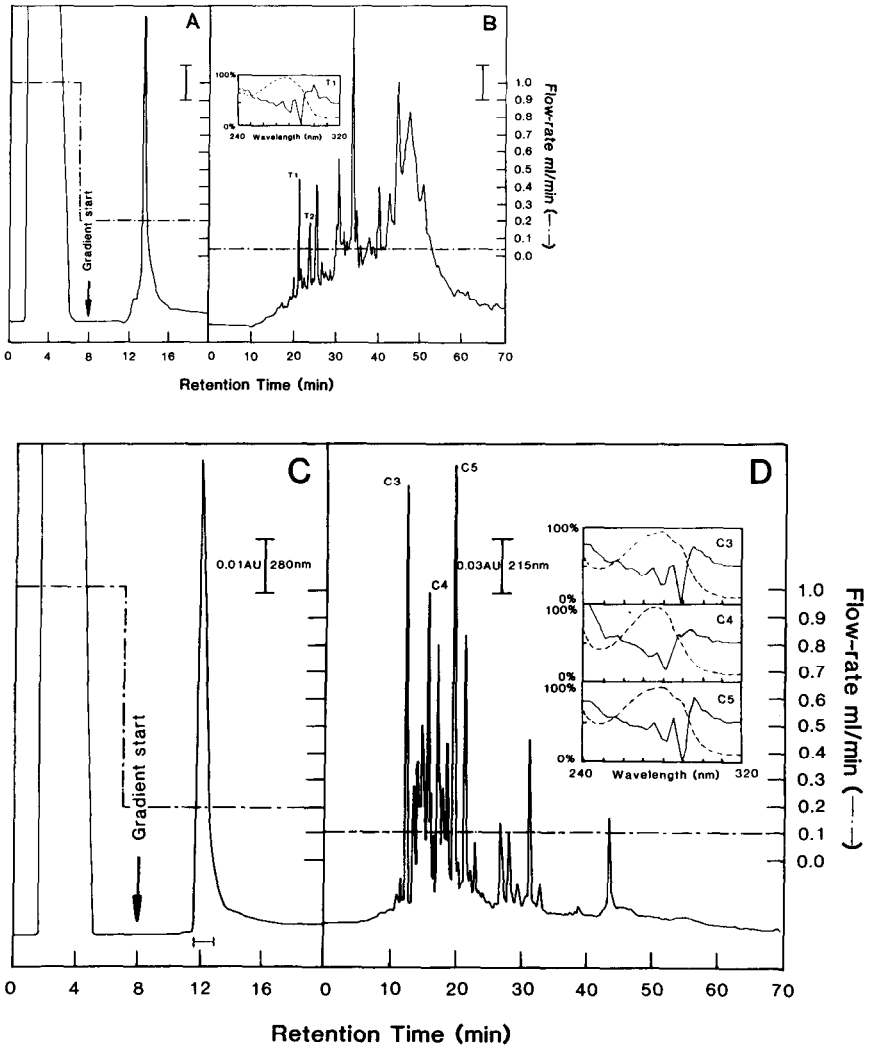

Fig. 5

(Continued on p. 358)

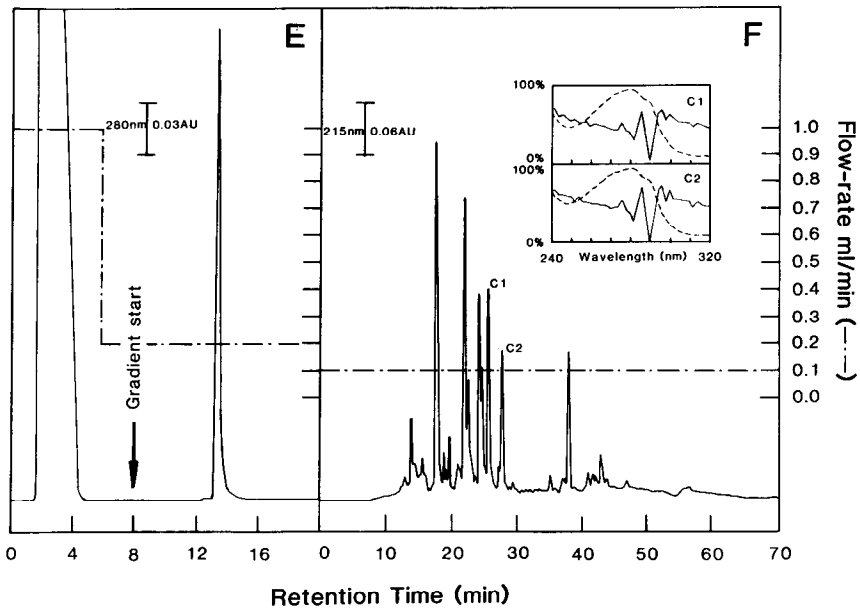

Fig. 5. Peptide mapping of proteins, recovered from detergent mixtures by inverse-gradient HPLC. Conditions for SDS-PAGE electrophoresis, electroblotting onto PVDF membrane, and visualisation with Coomassie Blue are described in Experimental. Coomassie Blue-stained proteins were eluted with 200 μl of 2% SDS–1% Triton X-100–0.1% dithiothreitol in 50 m*M* Tris–HCl buffer, (pH 9.0). Inverse-gradient HPLC conditions are given in Fig. 3. (A) Inverse-gradient HPLC of Coomassie Blue-stained β-lactoglobulin (6 μg) in 200 μl of 2% SDS–1% Triton X-100 elution buffer. Chromatographic conditions: column, Brownlee C_8 VeloSep (40 × 3.2 mm I.D.); the sample was loaded at 1 ml/min; the column was developed at 200 μl/min with a 5-min linear gradient from 0–100% B where eluent A = 90% aq. 1-propanol and eluent B = 50% aq. 1-propanol, containing 0.4% (v/v) trifluoroacetic acid. (B) Separation of a peptic digest of β-lactoglobulin, recovered from (A). For conditions of digestion see Experimental. Chromatographic conditions: column, Brownlee RP-300 (50 × 1.0 mm I.D.); the column was developed with a linear 60-min gradient from 0–100% B, where eluent A = 0.1% (v/v) aq. trifluoroacetic acid and eluent B = 60% aq. acetonitrile, containing 0.085% (v/v) trifluoroacetic acid. Peptides selected for sequence analysis are indicated. (C) Inverse-gradient HPLC of Coomassie Blue-stained S-carboxymethyl-lysozyme (g-type) (7 μg) from the Black Swan[45]. Conditions were the same as in (A). (D) Separation of peptic peptides of S-carboxymethyl-lysozyme (g-type) on a Brownlee RP-300 column (30 × 2.1 mm I.D.). Conditions were the same as in (B). (E) Inverse-gradient HPLC of Coomassie Blue-stained S-carboxymethyl S_7-glycoprotein (15 μg). Conditions were the same as in (A). (F) Separation of chymotrypsin peptides of S-carboxymethyl-S_7 glycoprotein on a Brownlee RP-300 column (30 × 2.1 mm I.D.). Conditions were the same as in (B). Selected peptides (identified by capital letters) were subjected to sequence analysis (Table VI). Spectral analysis of these peptides, obtained using a diode-array detector during elution, are illustrated in the insets. The absorption spectra have been normalized to relative absorbance on a scale of 0–100%. Zero-order-derivative spectra (– – –); second-order-derivative spectra (——).

aromatic amino acids. This is particularly useful if amino acid sequence data are required for the purpose of designing DNA probes, since tryptophan, with its unique codon, is readily identifiable by this means. An inspection of the spectra obtained in this study (Fig. 5, insets) reveals that all of the selected peptides contain aromatic amino acids, as judged by their absorption peaks in the range 270–300 nm. Enhancement of resolution by second-order-derivative spectroscopy reveals an

TABLE VI

SEQUENCE ANALYSIS OF PEPTIDES, ISOLATED BY SDS-PAGE ELECTROBLOTTING/ INVERSE-GRADIENT HPLC/RP-HPLC

Proteins (15 μg) were electrophoresed on SDS-PAGE and then electroblotted onto PVDF membranes (*cf.* Experimental). After visualisation with Coomassie Blue R250, protein spots were excised, placed in an Eppendorf tube and passively eluted for 30 min at 25°C with 2 × 100 μl 2% SDS–1% Tritons X-100–0.1% dithiothreitol–50 m*M* Tris–HCl buffer (pH 9.0). Protein recoveries, determined by amino acid analysis, were *ca.* 6 μg. Proteins were recovered from the detergent eluent by inverse-gradient reversed-phase HPLC, digested with either pepsin or chymotrypsin and the resultant peptides fractioned by microbore column HPLC (see Fig. 5). Selected peptides were subjected to sequence analysis.

Sample	*Initial yield in 1st cycle (pmol)*	*Sequence*	*Sequence matched*
β-Lactoglobulin:			
Peptide T1	14	Q K V A G T W	Residues 13–19
Peptide T2	13	Y V E E L K P T P E	Residues 42–51
S_7-Glycoprotein:			
Peptide C1	150	T I H G L W P D D V S T	Residues 29–40
Peptide C2	113	V L Q W P T A F	Residues 8–15
Lysozyme (g-type):			
Peptide C3	75	Q V D K R S H K P Q G T W	Residues 95–107
Peptide C5	104	K K F P S W T K D Q W	Residues 129–139

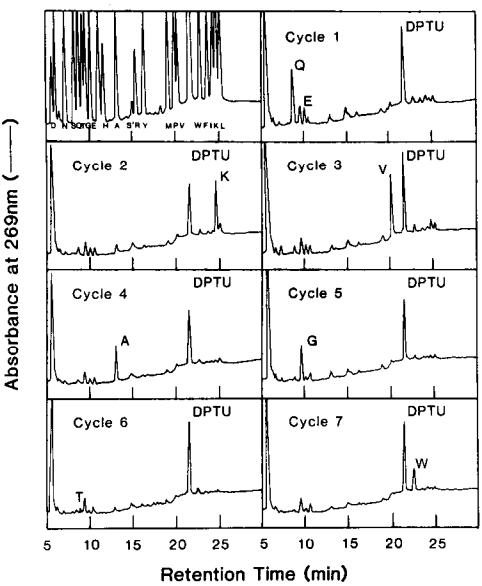

Fig. 6. Sequence analysis of peptic digest Peptide T1 from electroblotted β-lactoglobulin. β-lactoglobulin (*ca.* 20 μg) was electrophoresed by SDS-PAGE, electrotransferred onto PVDF, visualized with Coomassie Blue, and then eluted from PVDF with 200 μl of 2% SDS–1% Triton X-100 in 50 m*M* Tris–HCl buffer (pH 9.0), containing 0.1% dithiothreitol. The recovery of β-lactoglobulin, determined by amino acid analysis, was 6 μg. The remaining portion of β-lactoglobulin (*ca.* 4.5 μg) was recovered from the detergent mixture by inverse-gradient HPLC (Fig. 5A), digested with pepsin, and the peptide digest was fractionated by microbore HPLC (Fig. 5B). Peptide T1 (Fig. 5B) was subjected to sequence analysis. The HPLC elution patterns are shown. The positions of the PTH amino acids, assigned in the chromatograms from cycles 1–7, are indicated by the one-letter notation. The elution profile of a calibration mixture of PTH amino acids (25 pmol) is shown in the top left panel. DPTU denotes the byproduct, diphenyl thiourea.

extremum at 290 ± 2 nm for peptides C1, C2, C3, C5, and T1 (Fig. 5) and an extremum at 280 ± 2 nm for peptides C1–C5, T1 and T2 (Fig. 5); these are characteristic of tryptophan[43]. A maximum at 280 ± 2 nm only (*e.g.*, peptide C4) is characteristic of tyrosine residues[43]. The presence of tryptophan residues in peptides C1–C3, C5, and T1 and a tyrosine residue in peptide T2 was confirmed by amino acid sequence analysis (Table VI).

In summary, we present here a novel procedure for obtaining internal amino acid sequence information for proteins resolved by either SDS-PAGE or 2D gel electrophoresis. By combining gel electrophoresis, electroblotting, and HPLC (in both reversed-phase and inverse-gradient elution modes) we can obtain internal sequence data for electroblotted proteins, isolated in the low-microgram range. In the absence of detergents, the inverse-gradient HPLC reported in this study should be applicable to the separation of proteins under conditions of high organic solvent concentrations.

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