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Two-dimensional electrophoresis as a complementary method of isolating peptide fragments of cleaved proteins for internal sequencing

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Abstract

To determine the primary structure of proteins, usually proteolytic enzyme digests are separated by reversed-phase high-performance liquid chromatography (HPLC) and each fraction is collected and sequenced. The results obtained by different cleavages are combined to reveal the entire sequence. However, there are many N-terminal-blocked proteins and/or *intact* proteins or their particular fragments that are not eluted from HPLC columns. Internal fragments of such proteins were successfully isolated by the use of two-dimensional electrophoresis, after digestion. Electroblotted spots were easily sequenced to identify those difficult fragments which could not be obtained using HPLC.

1. Introduction

The complete amino acid sequence of proteins can usually be determined as follows. Initially, proteolytic enzyme digests of a protein are separated by reversed-phase high-performance liquid chromatography (HPLC), then each fraction is collected and sequenced. The partial sequences obtained by different cleavages are compared and overlapped to reveal the entire sequence. However, there are many N-terminal-blocked proteins that cannot undergo the Edman degradation. In addition, there are intact proteins, or their particular fragments, that cannot be separated or eluted from HPLC columns because of aggregation and adsorption. Sequence information cannot be obtained in these instances. This paper describes the isolation and identification of fragment peptides that allows the determination of their amino acid sequences, by the use of two-dimensional electrophoresis followed by electroblotting, as a complementary method to HPLC of protein digests.

2. Experimental

2.1. Materials

Trypsin, *Staphylococcus aureus* V8 protease and endoproteinase Lys-C were of sequence grade from Boehringer-Mannheim (Mannheim, Germany). Acrylamide and sodium dodecyl sul-

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phate (SDS) were obtained from Bio-Rad Labs. (Richmond, CA, USA), N,N'-methylenebisacrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium peroxodisulphate (APS), Pharmalyte 3–10, Ampholine 3.5–10, Ampholine 5–8, low-molecular-mass marker and protein isoelectric point (p*I*) marker from Pharmacia–LKB Biotechnology (Uppsala, Sweden), glycine, glycerol, acetic acid, Coomassie Brilliant Blue R-250, Nonidet P-40, methanol, ethanol, 2-mercaptoethanol and phosphoric acid from Nacalai Tesque (Kyoto, Japan), 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), 3-(cyclohexylamino)-1-propanesulphonic acid (CAPS) and human serum albumin (HSA) from Sigma (St. Louis, MO, USA), lysyl endopeptidase, *Achromobacter* protease I, hydrochloric acid, sodium hydroxide, bromophenol blue, formic acid, cyanogen bromide (CNBr) and phenylthiohydantoin (PTH-) amino acid standards from Wako (Osaka, Japan), urea from Fluka (Buchs, Switzerland) and a polyvinylidene difluoride (PVDF) membrane from Pall (New York, NY, USA). A protein from *Penicullium camembertii* (LPC) [1] was a generous gift from Dr. K. Isobe (GBF, Braunschweig, Germany).

2.2. Digestion

S-Carboxymethylated HSA was cleaved with CNBr according to ref. 2 and the digests were dissolved in the lysis buffer [3] at a concentration of 1.32 mg/ml. LPC was S-pyridylethylated, digested by trypsin and lyophilized. The resulting digests were dissolved in the lysis buffer at a concentration of 2 mg/ml and stored at −20°C until used.

2.3. HPLC separation and characterization of fragment peptides

Digests of LPC obtained using trypsin, endoproteinase Lys-C and *Staphylococcus aureus* V8 were lyophilized, separated as described in ref. 4 and characterized by sequencing and amino acid analysis.

2.4. Electrophoresis

Two-dimensional electrophoresis was performed using a TEP-2 instrument manufactured by Shimadzu (Kyoto, Japan). This apparatus [5] allows fully automated transfer from isoelectric focusing, the first-dimensional electrophoresis (1-DE) gel, to SDS polyacrylamide gel electrophoresis (PAGE), the second-dimensional electrophoresis (2-DE) gel. Running conditions and the transfer from 1-DE to 2-DE were performed automatically and precisely under the control of a microprocessor. The 1-DE gel was 1.5 mm in diameter and 140 mm long and the 2-DE gel was 160 mm long (separation gel 135 mm, stacking gel 25 mm), 160 mm wide and 1.5 mm thick. The temperature during electrophoresis can be accurately and directly regulated by the specially insulated metal plate and is independent of the ambient temperature. The composition of 1-DE gel was 9 *M* urea–6% Pharmalyte 3–10 (for HSA)–1.3% Ampholine 3.5–10–1.7% Ampholine 5–8 (for LPC)–5% T, 5% C–0.033% APS–0.067% TEMED. The cathode buffer for 1-DE was 0.02 *M* NaOH and the anode buffer was 0.085% H_3PO_4. Molecular mass and p*I* were calibrated prior to the sample analyses by the present apparatus. At 10°C, with a prerun at 200 V for 0.5 h, 1000 V for 4.8 h, followed by 1200 V for 1 h (for HSA), and a pre-run at 200 V for 1.0 h, followed by 1300 V for 7.0 h (for LPC), were performed in 1-DE, in which the gels were equilibrated in a solution of 62.5 m*M* Tris–5% 2-mercaptoethanol–2.3% SDS for 15 min (for HSA), 0.01 *M* H_3PO_4–5% 2-mercaptoethanol–2.5% SDS–8 *M* urea for 10 min (for LPC) *in situ*. With HSA, 2-DE was carried out according to Laemmli [6], and with LPC, 2-DE was carried out according to Swank and Munkres [7], which is suitable for peptide separations [8].

2.5. Electroblotting

Electroblotting onto the PVDF membrane was carried out at a constant 7 V/cm for 4.0 h at 4.0°C using Transphor TE42 (Hoeffer Scientific Instruments, San Francisco, CA, USA), with an electroblotting buffer consisting of 10 m*M* CAPS

(pH 11.0) containing 10% methanol and the pH was adjusted with 2 *M* NaOH. After blotting, the membrane was stained for 30 s in 0.2% Coomassie Brilliant Blue R-250 in 50% methanol–10% acetic acid and rapidly destained with 45% methanol–7% acetic acid.

2.6. Sequence analysis

Sequence analyses were performed using a PPSQ-10 gas-phase sequencer (Shimadzu), which allows highly sensitive isocratic elution for PTH-amino acid analysis [9]. The excised membrane was inserted directly in the reaction chamber of the sequencer. PTH-amino acid determination was performed by chromatogram subtraction in order to eliminate carry-over and background effects. The resulting PTH-amino acids were analysed by reversed-phase HPLC using a Wako-Pak WS-PTH column (250 mm × 4.6 mm I.D.) with 20 m*M* sodium acetate buffer (pH 4.7)–acetonitrile (60:40, v/v) containing 0.014% (w/w) of SDS at a flow-rate of 1.0 ml/min and detection at 269 nm.

3. Results and discussion

The present strategy allows the characterization of fragment peptides by sequence analysis, after isolation using two-dimensional electrophoresis followed by electroblotting. Chemically cleaved HSA was separated using TEP-2. The blotted PVDF membrane is shown in Fig. 1. Spot A (p*I* 6.2, relative molecular mass 27 000) was excised and sequenced to give an internal sequence of HSA from position 447, as indicated in Table 1. (Position 446 of HSA is a Met residue.) Forty residues were easily determined in a single run.

The digests of LPC, with different enzymes, were separated by reversed-phase HPLC and each peak was collected. All peaks were subsequently sequenced and analysed for their amino acid composition [4] and the entire sequence of LPC was thus determined. However, the results from mass spectrometry [10] and amino acid composition analysis of intact LPC were different from the above result. The fragments corresponding to positions 49–81 could not be found from digests separated by HPLC.

Table 1
Internal sequence obtained from spot A in Fig. 1 and amounts of PTH-amino acids (pmol)

1	2	3	4	5	6	7	8	9	10
Pro	(Cys)	Ala	Glu	Asp	Tyr	Leu	Ser	Val	Val
55.2		67.7	80.1	51.5	56.9	52.4	11.2	42.1	47.3
11	12	13	14	15	16	17	18	19	20
Leu	Asn	Gln	Leu	(Cys)	Val	Leu	Glu	His	Lys
37.3	36.7	30.0	37.7		32.6	31.4	17.4		25.0
21	22	23	24	25	26	27	28	29	30
Thr	Pro	Val	Ser	Asp	Arg	Val	Thr	Lys	(Cys)
	15.1	21.5		12.7		14.2	6.9	14.8	
31	32	33	34	35	36	37	38	39	40
(Cys)	Thr	Glu	Ser	Leu	Val	Asn	Arg	Arg	Pro
		14.3		9.7	9.7	8.7			

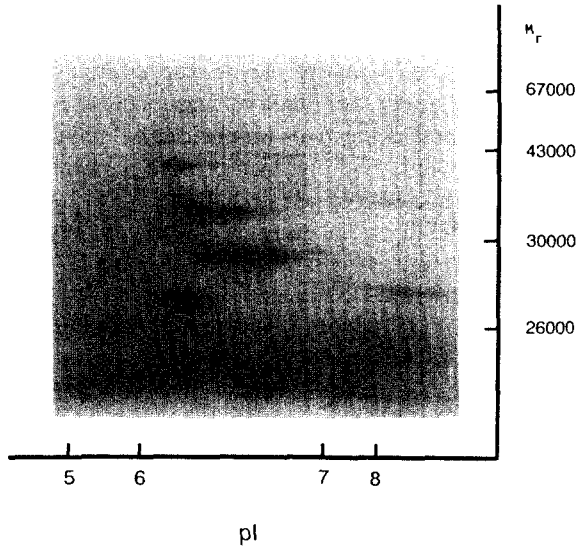

Fig. 1. Separated CNBr fragments of HSA on PVDF membrane. Spot A was excised and sequenced.

The tryptic digests of the S-pyridylethylated LPC were then separated by two-dimensional electrophoresis and electroblotted onto the PVDF membrane. Four spots were clearly observed (Fig. 2), excised and sequenced. Sequencing of spots A and B is summarized in Table 2. Several

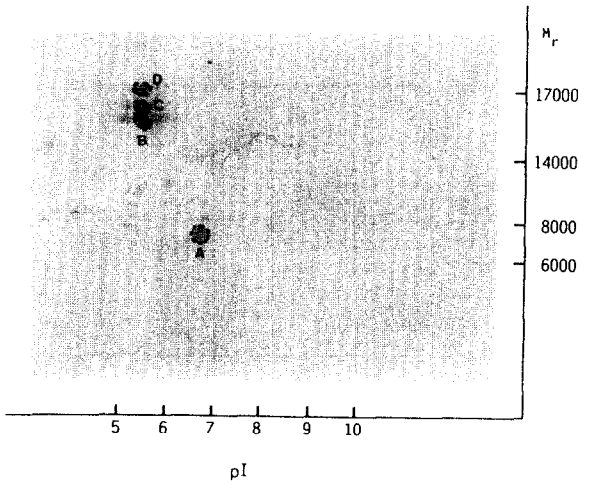

Fig. 2. Blotted membrane of tryptic digest of LPC after two-dimensional electrophoresis. Spots A, B, C and D were excised and sequenced.

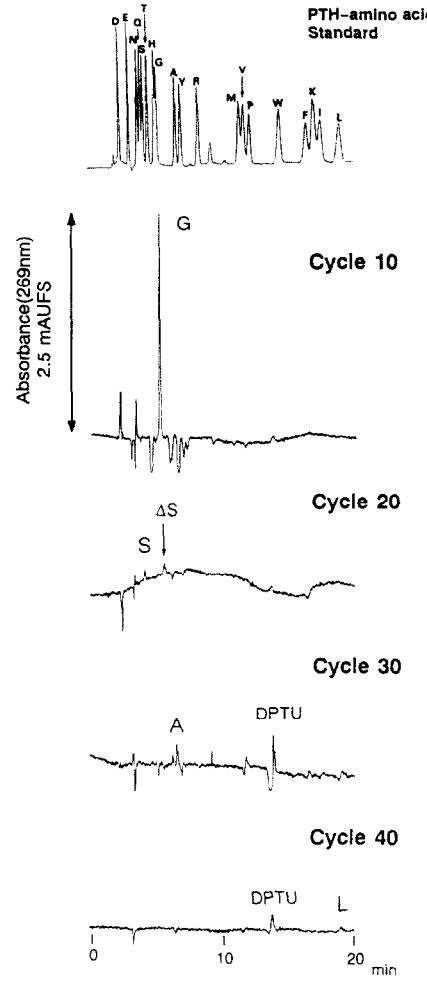

Fig. 3. Chromatograms of PTH-amino acid in the sequence analysis of spot B in Fig. 2.

chromatograms of the PTH-amino acids in the sequence analysis of spot B are shown in Fig. 3. Spots C and D gave the same sequence at the N-terminus as obtained from spot B, which was the sequence of positions 39–86 in the pyridylethylated LPC (Fig. 4). It seems that tryptic digestion of this region was only partially completed and the sequence of this region is resistant to trypsin, presumably owing to the conformation, and this leaves the fragments attached to the stationary phase of the column used for separation. The complete amino acid sequence of LPC has thus been determined.

Table 2
Internal sequence with amounts of PTH-amino acids (pmol) of LPC obtained from A, which gave positions 88–117, and B, which gave positions 39–86, in Fig. 4

Spot	1	2	3	4	5	6	7	8	9	10
A	Asn	Trp	Val	Ala	Asp	Ala	Thr	Phe	Val	His
	125	51	93	94	85	84	23	78	59	20
	11	12	13	14	15	16	17	18	19	20
	Thr	Asn	Pro	Gly	Leu	PEC	Asp	Gly	PEC	Leu
	16	50	38	31	39	24	31	22	17	22
	21	22	23	24	25	26	27	28	29	30
	Ala	Glu	Leu	Gly	Phe	Trp	Ser	Ser	Trp	Lys
	22	10	17	11	14	5	1	1		4
	1	2	3	4	5	6	7	8	9	10
B	Gly	Asn	PEC[a]	Pro	Glu	Val	Glu	Ala	Thr	Gly
	51	58		35	47	47	37	41	17	24
	11	12	13	14	15	16	17	18	19	20
	Ala	Thr	Val	Ser	Tyr	Asp	Phe	Ser	Asp	Ser
	33	14	27	6.4	17	21	17	4.3	15	6.1
	21	22	23	24	25	26	27	28	29	30
	Thr	Ile	Thr	Asp	Val	Ala	Gly	Tyr	Ile	Ala
	6.7	4.6	4.3	9.5	8.2	9.3	6.9	5.6		9.3
	31	32	33	34	35	36	37	38	39	40
	Val	Asp	His	Thr	Asn	Ser	Ala	Val	Val	Leu
	6.6	6.1	0.7		6.4			4.7	5.3	
	41	42	43	44	45	46	47	48	49	50
	Ala	Phe	Arg	Gly	Ser	Tyr	Ser	Val		

Spots C and D in Fig. 2 gave the same sequence as spot B, positions 39–64 of LPC [1].
[a] PEC = S-pyridylethylated cysteine.

4. Conclusions

Two-dimensional electrophoresis is a powerful complementary method for separating peptides and proteins, especially proteins which are difficult to separate by reversed-phase HPLC. The present strategy is useful for determining partial and internal sequences, especially of N-terminal-blocked proteins and for intact proteins or their particular fragments, which are not eluted from HPLC columns. Further, the excised spots on the PVDF membrane can also be used for amino acid analysis after direct hydrolysis [11]. These partial sequences can be used for DNA probes and allow the determination of the entire amino acid sequence of the proteins from the cDNA.

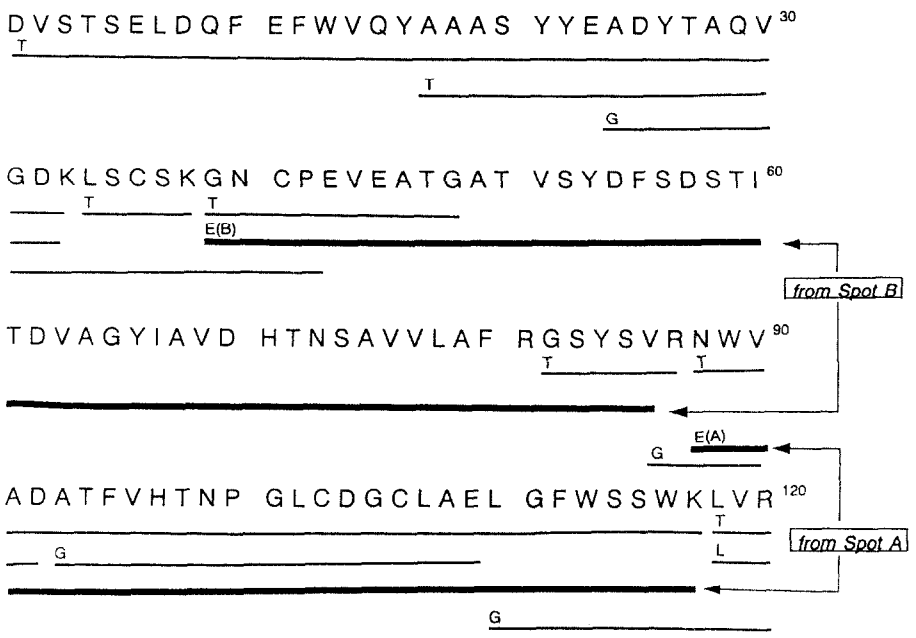

Fig. 4. Sequence of the present LPC determined by the present method. The prefixes T, L and G denote digests of trypsin, endoproteinase Lys-C and *S. aureus* V8 protease, respectively, determined by sequencing after fractionation by reversed-phase HPLC. E(A) and E(B) indicate sequences obtained by the present method from spots A and B, respectively, in Fig. 2.

5. References

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