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Capillary column high-performance liquid chromatographic–electrospray ionization triple-stage quadrupole mass spectrometric analysis of proteins separated by two-dimensional polyacrylamide gel electrophoresis Application to cerebellar protein mapping

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Abstract

A method is presented for the structural characterization of proteins separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The method includes separation of a protein mixture by 2D-PAGE, recovery of proteins from the gel spots revealed by copper staining and analysis of the proteins by triple-stage quadrupole mass spectrometry using an electrospray ionization interface (ESI-TSQMS). Prior to the mass spectrometric analysis, the extracted proteins were passed through a small reversed-phase column (10 × 4.0 mm I.D.) to remove salts and gel-derived contaminants and then introduced into the mass spectrometer through a reversed-phase capillary column with 0.25 mm I.D. Application of the method to the analysis of rat cerebellar proteins suggests that the molecular mass could be accurately determined with sub-picomole amounts of protein samples derived from one or two 2D gels. The method was also useful for peptide mapping and determination of amino acid sequences of proteins micro-prepared from the 2D gel. Because 2D-PAGE has an excellent resolving power in protein separation and because capillary LC–ESI-TSQMS provides structural information with very small amounts of samples, the combined system of 2D-PAGE and capillary LC–ESI-TSQMS described here should allow wide applications to molecular studies of genes and proteins, such as identifications of protein spots on 2D gels, confirmation of gene/protein sequences and analysis of post-translational modification of proteins present naturally in tissue/cell extracts or expressed by recombinant DNA techniques.

Keywords: Liquid chromatography–mass spectrometry; Detectors, electrophoresis; Peptide mapping; Proteins

1. Introduction

Among currently available techniques, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is the most powerful tool for protein separation. Because of its high resolution it is suitable for use in the total analysis of complex protein mixtures such as tissue or cell extracts, and the resulting electropherograms are often utilized as protein or polypeptide maps of par-

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ticular tissues or cells [1–5]. In general, the analysis of proteins by 2D-PAGE provides the physico-chemical parameters of separated proteins such as an isoelectric point (p*I*) and an approximate molecular mass, but does not provide direct sequence information. The assignment of protein spots on the 2D-gel map usually requires standard proteins for co-electrophoresis or their specific antibodies for immunochemical identification.

Attempts have been made to utilize 2D-PAGE for preparative purpose [6,7], particularly to obtain partial protein sequences, as the sequence information is valuable for the identification of proteins on gene/protein databases or for the design of gene probe sequences in molecular cloning. In most of these methods, protein spots separated by 2D-PAGE were digested in the gel matrix or after transfer to the polymer membranes, and the generated fragments were analysed on a conventional sequence analyser based on Edman chemistry. The amount of protein required for this process is in the range 10–100 pmol, depending on the sensitivity of the sequence analyser used, and this limits the application of the method to protein samples which are relatively abundant in tissues and cells.

With the development of methods of "soft ionization" such as fast atom bombardment, electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), mass spectrometry has recently emerged as a useful tool for the analysis of biopolymers such as proteins and nucleic acids [8,9]. In particular, ESI triple-stage quadrupole mass spectrometry (TSQMS) and MALDI time-of-flight mass spectrometry (TOFMS) are the focus of interest in protein chemistry as these methods could be rapid and sensitive tools for the determination of accurate molecular masses and amino acid sequences of proteins, and also for the analysis of post-translational modifications of proteins such as glycosylation and phosphorylation [10,11]. For example, it has been reported that the amino acid sequence of femtomole levels of peptide samples could be determined by MALDI-TOFMS using a post-source decay (PSD) technique [12]. At the current level of development, however, the PSD analysis of MALDI-TOFMS still requires a purified peptide which has previously been separated by LC or capillary electrophoresis. On the other hand, ESI-TSQMS could directly be connected to LC or capillary electrophoresis systems. Therefore, a combined system of LC and ESI-TSQMS allows on-line structural analysis of proteins and peptides in a crude mixture.

Here, we describe a method for the analysis of proteins separated by 2D-PAGE on a capillary column (0.25 mm I.D.) using LC–ESI-TSQMS. To prepare a protein sample efficiently from 2D gel we employed copper staining, and the extracted protein was purified on a reversed-phase precolumn before the protein or its proteolytic fragments were introduced into the capillary LC–EST-TSQMS system. We applied this method to the determination of the molecular masses and amino acid sequences of proteins detected on a polypeptide map of rat cerebellum.

2. Experimental

2.1. Materials

Reagents for electrophoresis were of the purest grade available from Wako (Osaka, Japan). Urea and a copper staining/destaining kit were purchased from Bio-Rad Labs. (Richmond, CA, USA), acetonitrile (chromatography grade) from Merck (Darmstadt, Germany) and trifluoroacetic acid (Sequanal grade) from Wako. All other reagents were of analytical-reagent grade from Wako, unless mentioned otherwise.

2.2. Preparation of rat cerebellar extracts

Wistar rat cerebella (wet mass 0.1 g) were homogenized in a Potter–Eljehem glass PTFE homogenizer with 0.3 ml of 8 *M* urea–1.2% Nonidet P-40 (NP-40)–6% 2-mercaptoethanol. The homogenate was mixed with 0.085 g of solid urea and centrifuged for 30 min at 15 000 *g*.

2.3. Two-dimensional electrophoresis

Two-dimensional electrophoresis was performed by a modification of O'Farrell's protocol [13]. The rat cerebellar extract containing 200 μg

of protein was focused for 17 h at 400 V and then for 1 h at 800 V on a prefocused 2.5-mm tube gel containing 8 *M* urea, 4% acrylamide, 2.5% carrier ampholytes [Ampholines pH 3.5–10 + pH 5–7 (1:1); LKB] and 1% Nonidet P-40. After focusing, the tube gel was equilibrated for 10 min in 2% SDS–5% 2-mercaptoethanol–10% glycerol solution and placed on a top of 1-mm thick, 14-cm wide, 10-cm high 12.5% polyacrylamide gel (pH 8.8) which was prepared under a 25-mm high stacking gel of 4% polyacrylamide (pH 6.8). Electrophoresis was performed for 5 h at 20 mA. The gel was immersed in Milli-Q-purified water for 1 min to remove excess SDS, and subsequently stained in 0.3 *M* $CuCl_2$ for 3 min [14]. The gel was used immediately for the preparation of protein samples as described below, or kept in Milli-Q-purified water at 4°C until used.

2.4. Preparation of protein samples for structural analysis

The protein spots were excised from a copper-stained polyacrylamide gel with a scalpel, transferred into polypropylene tubes and destained twice in 10 volumes of 25 m*M* Tris–Gly (pH 8.3) for 10 min and in 12.5 m*M* Tris–Gly (pH 8.3) for 10 min. The proteins were then extracted from the gel pieces. Of the two methods generally used for this purpose, electroelution and diffusion-based extraction, we employed the diffusion technique because it allows the simultaneous handling of many protein spots separated by 2D-PAGE. The gel pieces were immersed in 10 volumes of the extraction buffer, 50 m*M* Tris–HCl (pH 8.8) containing 50 m*M* EDTA and 0.1% SDS, and the proteins were extracted overnight at room temperature by end-over-end rotation (100 rpm).

The extracts thus obtained were loaded on a small Pnenyl-5PW RP reversed-phase column (10 × 4.0 mm I.D.) (Tosoh, Tokyo, Japan) and washed with 20% CH_3CN–0.1% TFA to remove salts, SDS, EDTA and gel-derived contaminants. The absorbed proteins were then recovered with a 15-min gradient of acetonitrile (20–60%) in 0.1% TFA at a flow-rate of 1.0 ml/min. Protein elution was monitored at 214 nm and the eluent was collected manually in a polypropylene tube. The eluent was concentrated to 15–30 μl under a gentle stream of nitrogen or under vacuum on a Speed Vac concentrator. The sample was analysed directly by capillary LC–ESI-TSQMS or, where necessary, subjected to proteinase digestion after the solution had been neutralized with small aliquots of 1.5 *M* Tris–HCl buffer (pH 8.8).

2.5. Capillary LC–ESI-MS

The molecular mass and the amino acid sequence of peptides were determined using the capillary LC–ESI-TSQMS system. The system consisted of a high-performance liquid chromatograph (Model 140 A; Applied Biosystems, Foster City, CA, USA) which was connected to a triple-stage quadrupole mass spectrometer equipped with an electrospray ionization interface (Model TSQ-700; Finnigan MAT, San Jose, CA, USA). The protein or peptide samples were injected on to a reversed-phase capillary column (100 mm × 0.25 mm I.D.) (Biotech Research, Saitama, Japan) packed with Capcell Pak C_{18} (particle size 3 μm; Shiseido, Tokyo), and eluted with a 40-min gradient from 0 to 80% acetonitrile in 0.1% trifluoroacetic acid. The pump was operated at 100 μl/min and the flow was split prior to the sample injector (Model 7725; Rheodyne) so that an optimum flow (2.5 μl/min) was delivered to the capillary column without a long delay time. The eluate was mixed postcolumn with 2-methoxyethanol–methanol–water–acetic acid (60:20:20:1) to assist ionization of peptides, and introduced to the electrospray interface of the mass spectrometer. The spectrometer was operated under the following conditions: electrospray voltage, 4.5 kV; heating capillary temperature, 200°C; electron multiplier voltage, 1200 V for peptide mapping and 1600 V for sequencing.

3. Results and discussion

3.1. Protein recovery from polyacrylamide gel

Among various staining reagents for proteins, Coomassie Brilliant Blue (CBB) is most commonly used for the detection of proteins in

polyacrylamide gel. We employed copper staining for the following reasons: (1) the staining procedure was simple and the sensitivity was equivalent to or several times higher than that of the conventional CBB method, e.g., copper staining required 10 min compared with 2 h for CBB staining; (2) CBB staining requires acidic methanol (50% methanol in 7% acetic acid) in order to fix proteins within the gel matrix, which would cause a low recovery of proteins on extraction because proteins are denatured, sometimes irreversibly, and are hardly solubilized even in the presence of SDS; although the extraction efficiencies of "acid-sensitive " proteins were improved by the use of low CBB concentrations at neutral pH, this method was less sensitive than the standard procedure; and (3) the CBB dye remained in the extracts co-eluted with some of the proteins upon subsequent reversed-phase HPLC.

To examine the extraction efficiency of the present method, four standard proteins (myoglobin, carbonic anhydrase, ovalbumin and serum albumin) were processed according to the extraction procedure described under Experimental; these proteins (1 μg each) were subjected to PAGE, copper-stained, extracted from the gel and purified by HPLC on a small reversed-phase column. The overall recovery of each protein was more than 60% (Table 1), suggesting that the method could be used for the micro-preparation of proteins in the gel matrix.

We applied this extraction method to the preparation of 32 rat cerebellar proteins separated by 2D-PAGE which have different molecular masses (M_r) (10 000–70 000) and acidity (pI 3.8–7.8). Of these proteins, 30 could be obtained with recoveries of 20–90% after the final reversed-phase chromatography for "brushing up" the extracted proteins, and structural information was obtained by mass spectrometry. Two proteins, both with relatively small M_r (30 000 and 19 000) and acidic pI (4.0), were not recovered under the conditions employed. However, we were not able to find a clear correlation between the extraction efficiency and the physico-chemical parameters of these proteins such as the M_r, hydrophobicity and pI, suggesting that some unknown factors have effects on the recovery of proteins from polyacrylamide gel.

Fig. 1 shows a typical result of "brush up" chromatography of a cerebellar protein (spot 259) extracted from the 2D gel. Following large UV-absorbing peaks in the flow-through fraction, a single protein peak appeared on gradient elution. The flow-through fraction contained large amounts of gel-derived, UV-absorbing impurities in addition to the buffer salts used for the extraction, and these materials interfered with the subsequent mass spectrometric analysis when the extracted protein samples were applied directly without reversed-phase HPLC. Fig. 2 shows the mass spectra of the extracted protein sample before and after reversed-phase HPLC.

Table 1
Recovery of standard proteins from copper-stained polyacrylamide gel

Protein	Recovery (%)
Human serum albumin	75
Ovalbumin	61
Carbonic anhydrase	80
Myoglobin	65

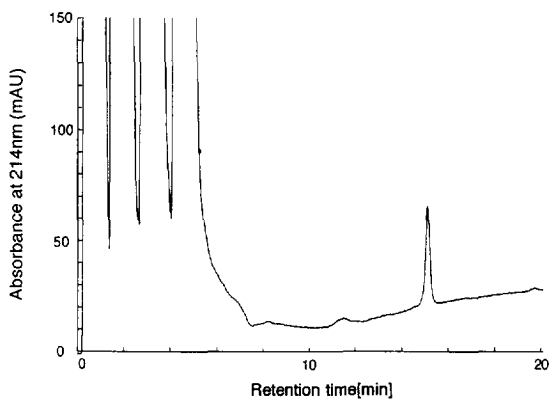

Fig. 1. Purification of spot 259 protein extracted from 2D gel. The spot 259 protein extracted from two copper-stained 2D-gels was applied to a reversed-phase precolumn (10 mm × 40 mm I.D.) of Phenyl-5PW RP. After washing the column with 20% acetonitrile in 0.1% trifluoroacetic acid, the adsorbed protein was eluted with a 15-min gradient of acetonitrile (20–60%) in 0.1% trifluoroacetic acid at a flow-rate of 1 ml/min.

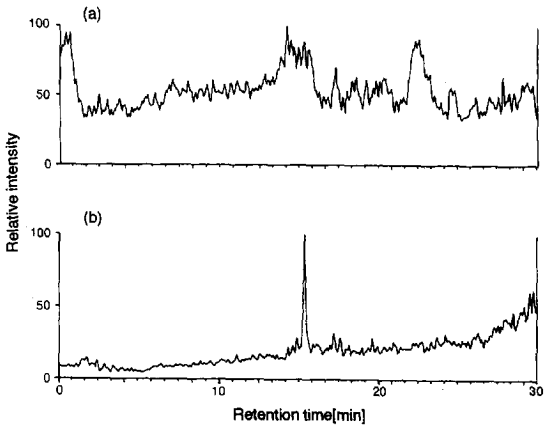

Fig. 2. Comparison of the capillary LC–MS of spot 259 protein extracted from 2D gel. The extracted protein (50 ng; 2.5 pmol) was subjected (a) directly to LC–MS analysis and (b) after purification by the reversed-phase HPLC, as shown in Fig. 1.

Without reversed phase HPLC, protein signals were hardly detected (Fig. 2a), even though a large portion of impurities was removed by capillary LC before the electrospray interface and not introduced into the mass spectrometer. In contrast, a single peak was detected for the extracted protein after it had been purified by the reversed-phase HPLC (Fig. 2b). Therefore, we found this purification step necessary for stable and highly sensitive LC–MS analysis.

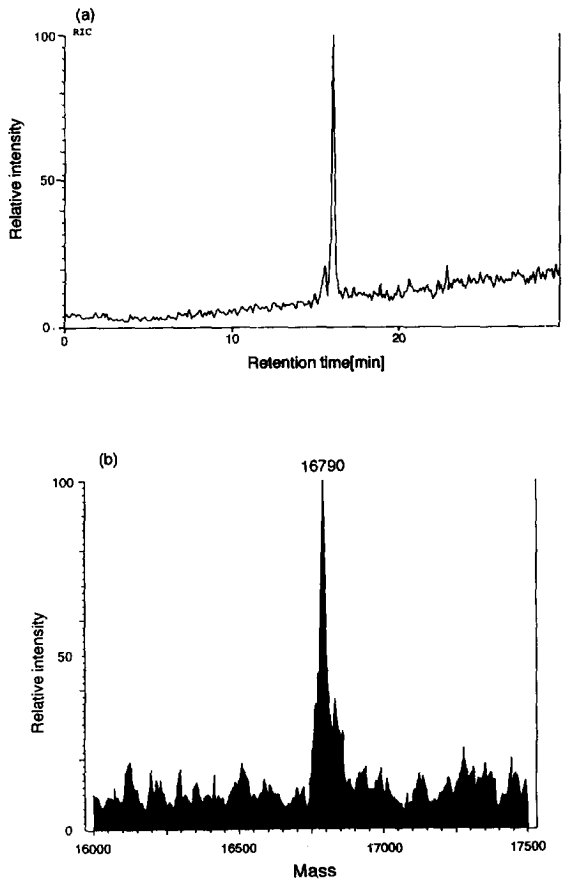

Fig. 3. Capillary LC–MS analysis of spot 259 protein (50 ng): (a) mass chromatogram and (b) deconvoluted mass spectrum.

3.2. Mass spectrometric analysis of proteins extracted from 2D gel

Molecular mass determination

Among 400 protein spots detected by copper staining of the 2D gel, we selected 20 spots with different molecular masses and acidity and the proteins were extracted as described above. Fig. 3 shows examples of (a) the mass chromatogram and (b) the deconvoluted spectrum of a protein (spot 259) extracted from a single 2D gel. The mass spectrometric analysis showed that spot 259 was composed of a single molecular species with a molecular mass of 16 790. Likewise, 18 spots examined gave equivalent spectra from which the mass values were estimated. Several spots, such as spot 199, contained more than two polypeptide species, which were resolved by reversed-phase capillary LC and their molecular masses were determined simultaneously by on-line LC–MS (Fig. 4). A further extended analysis of spot 199 indicated that this contained a 14-3-3 family of proteins, the separation of which could have been achieved only by reversed-phase HPLC [15]. This is one of the advantages of the present system, in which the resolution of proteins in 2D-PAGE is supplemented by reversed-phase LC as the method is based on a separation principle different from that of 2D-PAGE.

Peptide mapping and primary structural analysis

A portion of the protein sample micro-prepared from the 2D gel was digested with TPCK–trypsin and the digest was analysed by the

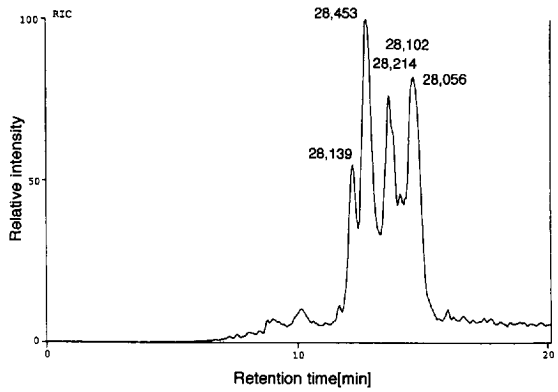

Fig. 4. Capillary LC–MS analysis of spot 199 protein (ca. 200 ng). Note that spot 199 was composed of several protein subspecies with similar molecular masses, which were separated by reversed-phase capillary LC.

capillary LC–MS system for peptide mapping and amino acid sequence determination. To achieve highly sensitive analysis with a small amount of the sample (ca. 5 pmol or below), we used a capillary column of 0.25 mm I.D. and employed postcolumn admixture of 2-methoxyethanol–methanol–water–acetic acid (60:20:20:1) to assist ionization and to obtain a stable baseline [26].

Fig. 5 shows an example of the mass chromatogram of the tryptic digest of spot 259. On the chromatogram 14 fragments were detected and their molecular masses were determined simultaneously by the LC–MS system. A further experiment, described below, identified that this spot, No. 259, was calmodulin, a ubiquitous calcium-binding protein of eukaryotic cells. Calmodulin consists of 148 amino acids and the tryptic cleavage should produce thirteen fragments [16]. These fragments were easily identified in the mass chromatogram of spot 259 based on the molecular mass of each fragment. The identified fragments included all of the fragments expected from the calmodulin sequence, except for dipeptide T6 (Mel–Lys), and

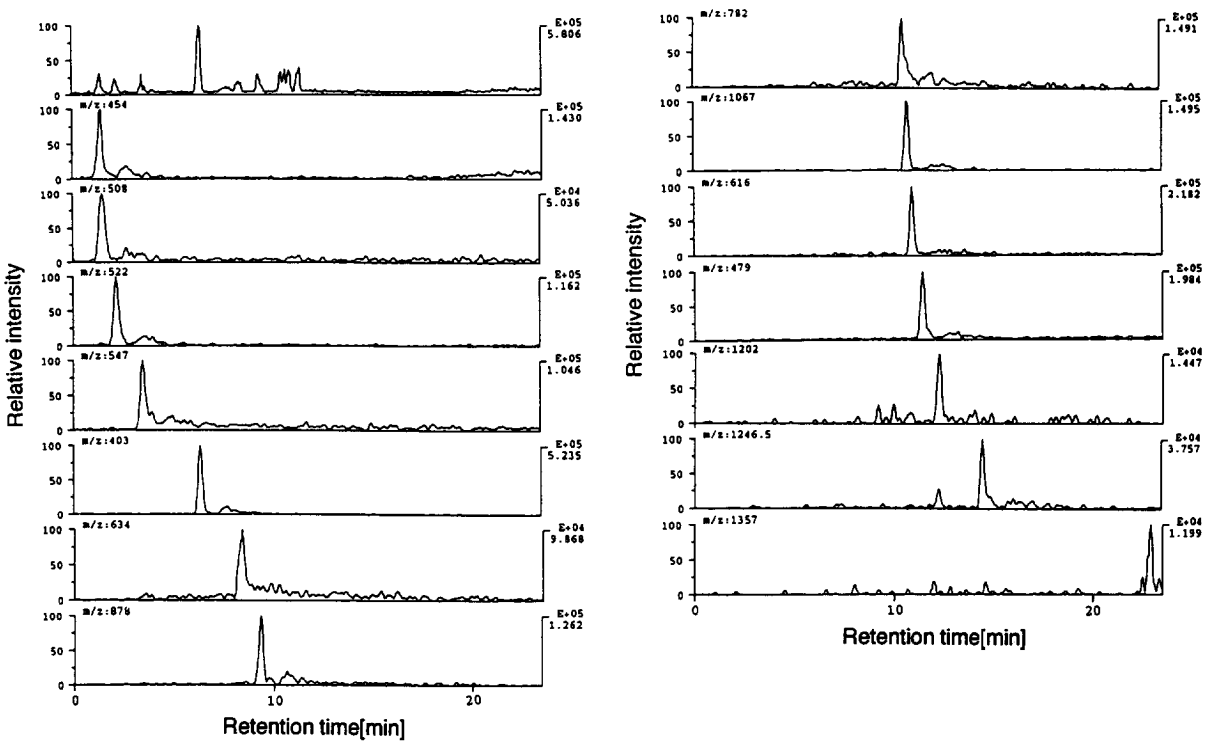

Fig. 5. Capillary LC–MS peptide map of the tryptic digest of the spot 259 protein. The tryptic digest (2.5 pmol) was analysed by capillary LC–MS. The selected ion chromatograms are shown.

T7 (free Lys), both of which were out of the mass range measured. An acetylated N-terminus and trimethyllysine resulting from post-translational modification in calmodulin [16] could also be detected by the present LC–MS analysis.

To determine the amino acid sequence by tandem mass spectrometry, one of the fragments detected in Fig. 5, marked T1, with a mass of 1563, was selected in the first quadrupole mass filter as one of the examples and was transported to the second quadrupole collision cell. The doubly charged peptide ion with m/z 782.5 was fragmented by collision-activated dissociation (CAD) and the product ions detected in third quadrupole mass spectrometry were analysed to obtain the sequence information. The resulting CAD spectrum is shown in Fig. 6. Because it was a tryptic peptide and had a positive charge due to the C-terminal Lys/Arg, y ion series signals were significantly detected in the CAD spectrum. The analysis of a series of product ions in Fig. 6 showed the sequence Ac-Ala–Asp–Gln–Ile/Leu–Thr–Glu–Glu–Gln–Ile/Leu–Ala–Glu–Phe–Lys. The computer-assisted retrieval of this sequence in the NBRF/PIR protein sequence database identified unequivocally that the spot 259 is calmodulin.

Table 2 summarizes the results of the present analysis, with the spot number, the apparent molecular mass determined by 2D-PAGE, the observed mass from LC–MS and, where identified, the assignment of the proteins and its average molecular mass calculated from the amino acid sequence. The proteins analysed in this study have a molecular mass range from 17 000 to 67 000. In general, however, the system is capable of analysing proteins with larger molecular masses, such as monoclonal IgGs with mass of 150 000 [27]. This suffices for the purpose of the present study, because most of the proteins separated by 2D-PAGE have molecular masses within 150 000. As shown in Table 2, the observed and calculated masses agreed to within 0.01% for calmodulin and beta 14-3-3 protein, whereas larger differences were observed for other proteins. The reasons for the observed differences are unclear; however, further extended LC–MS analyses of some of these proteins

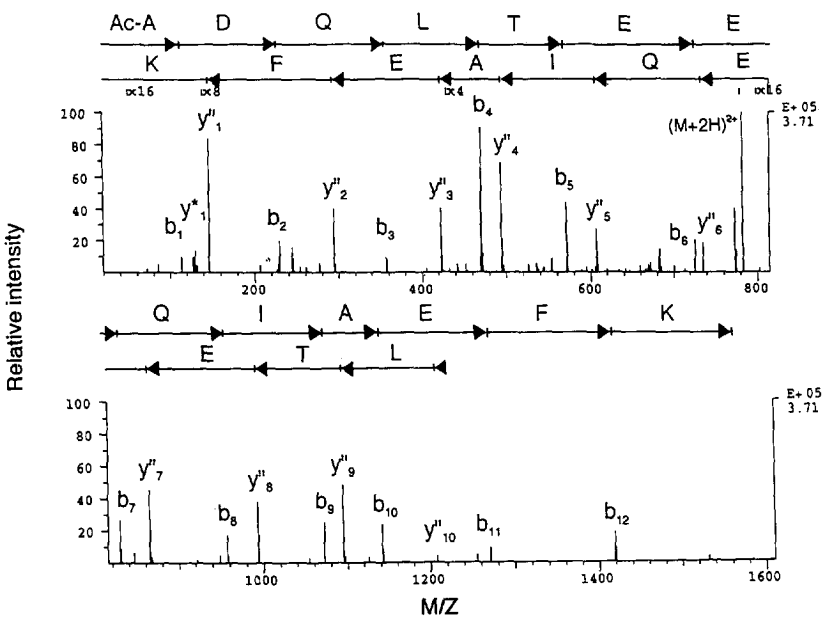

Fig. 6. CAD spectrum of a tryptic fragment of the spot 259 protein. A doubly charged ion of the tryptic fragment (m/z 782.5) was analysed by capillary LC–MS–MS. Ions of type y", b and a are labelled. The amino acid sequence reconstructed from these product ions is shown by the usual single letter code.

Table 2
Molecular mass determination of the proteins extracted from 2D gel

Spot No.	M_r				Identification
	2D-PAGE	Observed	Calculated	Δm[a]	
47	66 000	68 564	65 905	2659	Albumin [17]
64	60 000	58 177			
75	50 000	52 525			
83	50 000	49 419			
86	47 000	47 494	47 110[b]	384	NNE [18]
103	45 000	22 037	21 532	505	NAP-22 [19]
119	43 000	42 948	42 576[c]	372	Creatine kinase [20]
121	43 000	47 562			
156	36 000	36 891			
174	35 000	35 125			
184	30 000	29 445	29 171	274	14–3–3ε [21]
		29 546			
199	27 000	28 056	28 054	2	14–3–3β [22]
		28 102			
		28 139			
		28 214	28 213	1	14–3–3γ [23]
		28 453			
201	25 000	25 264	24 776	488	PGP9.5 [24]
233	21 000	20 855	20 852[b]	3	PEBP[d]
234	21 000	21 926	21 783	143	TSA[e]
246	18 000	17 201	17 199[b]	2	Stathmin [25]
247	18 000	17 278	17 279[b]	1	Stathmin
259	17 000	16 790	16 790[b]	0	Calmodulin [16]

[a] ΔM = difference between observed and calculated molecular masses.
[b] Estimated under the assumption that the mature protein lacks the initial methionine and has an acetyl group at the N-terminus.
[c] Estimated under the assumption that the mature protein lacks the initial methionine.
[d] Genbank accession number RNPEABP.
[e] Genbank accession number RNU06099.

suggest that the differences could be due to post-translational modifications in, for instance, phosphatidylethanolamine-binding protein (spot 233) and stathmin (spot 246, 247).

4. Conclusion

A method has been presented for the structural characterization of proteins micro-prepared from 2D gels. We found that (1) most of the proteins in the 2D gel matrix could be extracted with reasonable recoveries from the copper-stained gel, (2) the purification of the extracted proteins by reversed-phase chromatography was essential for the highly sensitive capillary LC–TSQMS analysis and (3) the protein sample thus obtained was suitable for subsequent structural determinations by mass spectrometry. The amount of protein required to obtain sequence information was in the sub-picomole to several hundred femtomole range, which was about ten times more sensitive than with conventional sequenator analysis. Another advantage of the present method is that the single LC–MS analysis of a protein digest allows the simultaneous determination of the amino acid sequence of 4–5 peptides within 3–4 h. One of remaining problem is an automated interpretation process from CAD signals to amino acid sequence with a computer algorithm. Although the method still requires improvement in several respects such as the recovery of some proteins from 2D gels, we conclude that the combined system of 2D-PAGE

and capillary LC–ESI-TSQMS would be useful for studies of various biological problems which require protein analysis.

References

[1] J.E. Celis, H.H. Rasmussen, P. Madsen, H. Leffers, B. Honore, K. Dejgaard, B. Gesser, E. Olsen, P. Gromov, H.J. Hoffmann, M. Nielsen, A. Celis, B. Basse, J.B. Lauridsen, G.P. Ratz, H. Nielsen, A.H. Anderson, E. Walbum, I. Kjaergaard, M. Puype, J. Van Damme and J. Vandekerckhove, Electrophoresis, 13 (1992) 893.

[2] C.S. Giometti, J. Taylor and S.L. Tollaksen, Electrophoresis, 13 (1992) 960.

[3] D.F. Hochstrasser, S. Frutiger, N. Paquet, A. Bairoch, F. Ravier, C. Pasquali, J.-C. Tissot, B. Bjellqvist, R. Vargas, R.D. Appel and G.J. Hughes, Electrophoresis, 13 (1992) 992.

[4] M. Yun, W. Wu, L. Hood and M. Harrington, Electrophoresis, 13 (1992) 1002.

[5] R.A. VanBogelen, P. Sankar, R.L. Clark, J.A. Bogan and F.C. Neidhardt, Electrophoresis, 13 (1992) 1014.

[6] P. Matsudaira, J. Biol. Chem., 262 (1987) 10035.

[7] G. Bauw, J. Van Damme, M. Puype, J. Vandekerckhove, B. Gesser, G.P. Ratz, J.B. Lauridsen and J.E. Celis, Proc. Natl. Acad. Sci. U.S.A., 86 (1989) 7701.

[8] P.R. Griffin, J.A. Coffman, L.E. Hood and J.R. Yates, Int. J. Mass Spectrom. Ion Processes, 111 (1991) 131.

[9] D.F. Hunt, J. Shabanowitz, J.R. Yates, P.R. Griffin and N.Z. Zhu, in C.H. McEwen and B.S. Larsen (Editors), Mass Spectrometry of Biological Materials, Marcel Dekker, New York, 1990, Ch. 8, p. 169.

[10] K. Matsuoka, K. Seta, Y. Yamakawa, T. Okuyama, T. Shinoda and T. Isobe, Biochem. J., 298 (1994) 435.

[11] H. Taniguchi, M. Suzuki, S. Manenti and K. Titani, J. Biol. Chem., 269 (1994) 22481.

[12] R. Kaufmann, B. Spengler and F. Lutzenkirchen, Rapid Commun. Mass Spectrom., 7 (1993) 902.

[13] P.H. O'Farrell, J. Biol. Chem., 250 (1975) 4007.

[14] C. Lee, A. Levin and D. Branton, Anal. Biochem., 166 (1987) 308.

[15] T. Isobe, T. Ichimura, T. Sunaya, T. Okuyama, N. Takahashi, R. Kuwano and Y. Takahashi, J. Mol. Biol., 217 (1995) 125.

[16] H. Kasai, Y. Kato, T. Isobe, H. Kawasaki and T. Okuyama, Biomed. Res., 1 (1980) 248.

[17] T.D. Sargent, M. Yang and J.J. Bonner, Proc. Natl. Acad. Sci. U.S.A., 78 (1981) 243.

[18] K. Sakimura, E. Kushiya, M. Obinata and Y. Takahashi, Nucleic Acids Res., 13 (1985) 4365.

[19] S. Maekawa, M. Maekawa, S. Hattori and S. Nakamura, J. Biol. Chem., 268 (1993) 13703.

[20] P.A. Benfield, L. Henderson and M.L. Pearson, Gene, 39 (1985) 263.

[21] P.H. Roseboom, J.L. Weller, T. Babila, A. Aitken, L.A. Sellers, J.R. Moffett, M.A. Namboodiri and D.C. Klein, DNA Cell Biol., 13 (1994) 629.

[22] M. Watanabe, T. Isobe, T. Ichimura R. Kuwano, Y. Takahashi and H. Kondo, Mol. Brain Res., 17 (1993) 135.

[23] Y. Kajimoto, T. Hashimoto, Y. Shirai, N. Nishino, T. Kuno and C. Tanaka, J. Biochem., 112 (1992) 28.

[24] V. Doye, F. Soubrier, G. Bauw, M.-C. Boutterin, L. Beretta, J. Koppel, J.S. Vandekerckhove and A. Sobel, J. Biol. Chem., 264 (1989) 12134.

[25] P.K. Bender, J.R. Dedman and C.P. Emerson, Jr., J. Biol. Chem., 263 (1988) 9733.

[26] H. Nakayama, K. Uchida, K. Seta and T. Isobe, J. Chromatogr. A, submitted for publication.

[27] H. Nakayama and K. Seta, unpublished results.