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Structural characterization of polypeptides and proteins by combination of capillary electrophoresis and ^{252}Cf plasma desorption mass spectrometry

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ABSTRACT

An efficient and sensitive method for the isolation and transfer of peptides and proteins from capillary zone electrophoresis separation for subsequent analysis by ^{252}Cf plasma desorption mass spectrometry was developed. Sample isolation on to nitrocellulose-coated targets for mass spectrometric analysis is performed by using a stainless-steel microtube pre-filled with aqueous buffer solution, to which the capillary end is connected, and the peptide is collected by applying a suitable transfer voltage according to the separation voltage. Low- and sub-picomolar sample amounts were isolated with high transfer efficiency and reproducibility, without the necessity for independent determination of electroosmotic flow-rates. Plasma desorption mass spectra of several peptides and proteins showed predominantly intact molecular ions; however, for several peptides partial oxidative modification was found which can be accounted for by the electrophoretic separation and/or transfer conditions. First applications to peptides and proteins show the feasibility of this off-line combination for primary structure characterization, such as by *in situ* chemical modification and enzymatic proteolysis reactions on the sample target prior to mass spectrometric analysis.

INTRODUCTION

Capillary electrophoresis (CE) has been developed in recent years as a powerful, high-resolution microanalytical separation method [1], which is currently finding growing attraction and application to the characterization of drug metabolites and biomacromolecules, such as peptides and proteins [2,3]. One of the key analytical features of CE is the high detection sensitivity in the pico- to femtomolar range, at high separation efficiency, obtained at very low flow-rates and small sample volumes of microcell UV or fluorescence detectors [4]. In contrast, the identification and structural characterization of separated compounds is at present a major problem with the CE method, particularly in the analysis of multi-component mixtures [5].

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The successful development of desorption–ionization methods of mass spectrometry (MS) in recent years such as fast atom bombardment (FAB-MS), ^{252}Cf plasma desorption (PD-MS) and, more recently, electrospray (ES-MS) and laser desorption (LD-MS) has permitted molecular mass determinations and structural analyses of biopolymers, particularly polypeptides and proteins up to and beyond M_r 100 000 [6–9]. Direct mass spectrometric methods have been successfully integrated in primary structure studies of proteins by combination with selective chemical and enzymatic modification, sequential degradation reactions and with proteolytic digestion procedures (peptide mapping) [10–12]. For example, the combination of PD-MS with Edman degradation has been successfully applied to primary structure studies of polypeptides and peptide mixtures [13]. A particular advantage of the PD-MS method has been the possibility of directly carrying out and analysing "*in situ*" chemical reac-

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tions on the nitrocellulose (NC) sample target, which has been developed as an efficient approach in structural studies, *e.g.*, by peptide mapping [14,15].

In this paper an isolation and transfer method for CE-separated polypeptides is described, which is feasible for direct subsequent analysis by PD-MS or other desorption–ionization mass spectrometric methods. Suitable "on-line" interfaces for the direct coupling of CE with ES-MS [16] and FAB-MS [17] have recently been reported, using either a coaxial sheath flow or a liquid-junction interface [18]. However, an efficient and practical "off-line" isolation and microtransfer procedure appeared to be advantageous in structural studies of polypeptides by providing the possibility for microsequencing and alternative analytical methods such as *in situ* reactions, thus complementing the mass spectral analysis [19]. An isolation procedure using a porous glass joint at the end of the CE capillary has been reported previously by Takigiku and co-workers [20,21], but this is technically demanding and requires independent determinations of electroosmotic flow-rates. In this study an isolation procedure similar to the fraction collector described by Rose and Jorgenson [5] was developed by using suitable micro-sample tubes for direct injection on to the PD-MS sample target. PD-MS studies of a variety of model peptides and small proteins revealed high transfer efficiencies. The straightforward feasibility of this approach is demonstrated by first application examples of structural analysis, in combination with sequential and proteolytic degradation. Further, PD-MS analyses of several peptides are described, showing the occurrence of structural (oxidative) modification, and degradation of phenylthiocarbamoyl–peptide adducts which have not been observed previously [20].

EXPERIMENTAL

Polypeptides

The following commercially available peptides and proteins were used: bradykinin (acetate salt), luteinizing hormone-releasing hormone (LHRH) (acetate), human angiotensin I (acetate), melittin and hen egg white lysozyme (HEL) from Sigma (St. Louis, MO, USA) and neurotensin (research grade) and bovine insulin from Serva (Heidelberg, Germany). The homogeneity and purity of all polypeptides were assessed by HPLC and mass spectral analysis, and was found to be ⩾95% except for melittin (*cf.*, Fig. 2).

Capillary electrophoresis

A CE-100 instrument (Grom, Herrenberg, Germany) equipped with an uncoated 50 μm I.D. fused-silica capillary was used. All separations were performed with 20 m*M* citric acid–15m*M* ammonium chloride (pH 2.2) buffer solutions in deionized water obtained from a Milli-Q system (Waters–Millipore), filtered through a 0.45-μm membrane filter and degassed by ultrasonication prior to use. The total length L of the capillary varied from 62 to 73.5 cm, while the length l from the beginning of the capillary to the UV detector cell was kept constant at 48.5 cm (Fig. 1). All CE separations were carried out at approximately 20°C at a potential difference of 20–25 kV and a current of 25–31 μA; electropherograms were obtained with a Linear UVIS-100 multiple-wavelength detector and recorded with a Shimadzu C6a integrator.

CE isolation and transfer for PD-MS analysis

For the isolation of peptides, the end of the capillary from the UV detector was plugged into a 5-μl stainless-steel sample tube or, alternatively, connected to a microbore stainless-steel block containing 2 μl of solvent; in both instances sample tubes were at ground potential (Fig. 1). CE migration times were determined with the integrator and subsequent sample isolation or transfer performed under identical voltage (20–25 kV) conditions but with a slightly reduced current. The isolation start time ($t_{i,s}$) and isolation time (t_i) were calculated from the beginning (t_1) and end (t_2) of the UV-detected peak (1% baseline) according to

$$t_{i,s} = t_1 L/l \quad (1)$$

$$t_i = (t_2 - t_1)(L/l)C \quad (2)$$

where L/l = ratio of total capillary length to capillary length to UV detector and C (1.05) = correction factor for the decreased current.

For subsequent PD-MS analysis the isolated sample solutions were transferred on to nitrocellulose-coated surfaces by means of a microlitre syringe.

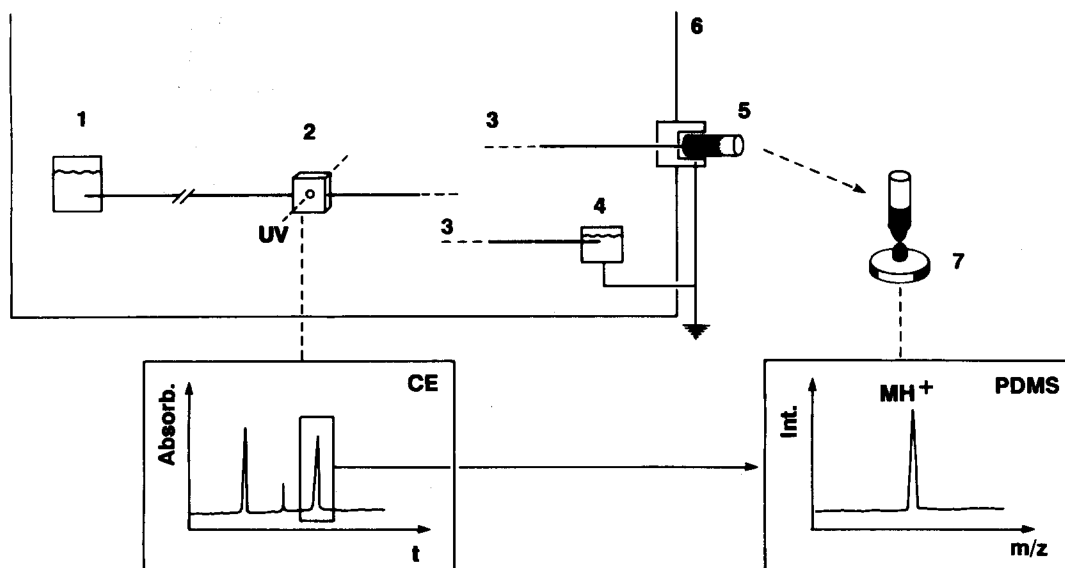

Fig. 1. Scheme of the CE isolation and transfer procedure for PD-MS analysis. Following the CE separation the capillary end is removed from the buffer reservoir (4) and connected to a stainless-steel tube or metal block (5) as described under Experimental. The isolation times for separated components are calculated from the migration times of the UV-detected peak. The collected sample solution is transferred on to the NC target with a microsyringe. 1 = Sample loading reservoir; 2 = UV detector cell; 3 = CE capillary; 4 = buffer reservoir; 5 = transfer capillary; 6 = insulated CE instrument housing; 7 = PD-MS sample target.

Mass spectrometry

Nitrocellulose (NC) targets for sample adsorption were prepared by electrospraying from an acetone solution as previously described [22]. Peptides isolated from CE into 2–5 μl of 0.1% trifluoroacetic acid (TFA) were allowed to adsorb for 3 min on the NC target, followed by twofold washing with 50 μl of 0.1% TFA and spin drying [23] for removal of salt contamination. PD-MS analyses were performed with a Bio-Ion/Applied Biosystems 20 K (Uppsala, Sweden) time-of-flight spectrometer as described [24], using an accelerating voltage of 16 kV.

In situ tryptic peptide mapping analysis

Experimental details of the *in situ* PD-MS peptide mapping analysis of proteins on the NC target surface have been described [12,25]. Disulphide linkages of lysozyme adsorbed on NC after CE isolation were cleaved by addition of 2 μl of a 80 m*M* dithiothreitol (DTT) solution in 50 m*M* NH_4HCO_3 (pH 8.3), and reduction was carried out for 30 min at 20°C under a microscope cover-slip. After removal of excess of DTT by washing with 50 m*M* NH_4HCO_3, proteolytic digestion was carried out for 30 min at 37°C under a microscope cover-slip by addition of 2 μl of TPCK-treated trypsin (Sigma) (0.1 mg/ml) in 50 m*M* NH_4HCO_3, and sample targets were prepared by spin drying for PD-MS analysis.

Edman–PD-MS analysis

Combined Edman–PD-MS analysis cycles of CE-separated polypeptides were performed by manual Edman degradation as described [13]. Edman coupling of angiotensin I was carried out by adding a solution (20 μg in 20 μl of water) to 20 μl of a 5% (v/v) solution of phenyl isothiocyanate (PITC) (sequencing grade; Pierce, Rockford, IL, USA) in pyridine, and the reaction was allowed to proceed for 45 min at 25°C with gentle shaking. After lyophilization to dryness the cleavage step was carried out with 20 μl of anhydrous TFA for 15 min at 20°C. The lyophilized cleavage product was dissolved in 40 μl of 50 m*M* NH_4HCO_3, an approximately 10% aliquot was withdrawn for CE and PD-MS analysis and the remaining solution was subjected to subsequent Edman degradation cycles.

RESULTS AND DISCUSSION

Capillary electrophoresis isolation and transfer method for ^{252}Cf plasma desorption mass spectrometry

The instrumental scheme used for isolation and transfer of CE-separated polypeptides to subsequent PD mass spectrometric analysis is shown in Fig. 1. An essential precondition for PD-MS analysis with high sensitivity is the application and adsorption of homogeneous peptide solutions of typically 1–10 μl on to the nitrocellulose-coated target surface [18,22], where final sample preparation can be carried out by spin drying [23] or additional washing steps for removal of buffer or residual salt contaminants [22]. Owing to the minimal liquid flow in CE separation [26], sample isolation was performed by connecting a stainless-steel microtube or metal block to the CE instrument housing, prefilled with a suitable solvent (typically 2–5 μl of 0.1% TFA), into which the end of the fused-silica CE capillary is fed (*cf.*, Fig. 1). Isolation times for CE-separated components were calculated from the ratio of total capillary length to the capillary length to the UV detector (see Experimental), and the migration times of the UV-detected peak. This procedure enables the reproducible isolation of peptides without independent determination of electroosmotic flow-rates and with a free choice of CE buffers and pH, in contrast to isolation by means of a porous glass joint [20].

An example for the isolation conditions thus obtained is shown in Fig. 2 by comparison of PD mass spectra of the polypeptide melittin before and after CE separation. The PD spectrum of melittin (Fig. 2A) yielded the most abundant protonated molecular ion $[M+H]^+$ at m/z 2849 together with the doubly charged $[M+2H]^{2+}$ ion, which is associated by a by-product ion at m/z 2878. The electropherogram (Fig. 2B) revealed the most intense peak at an average migration time of 8.6 min, accompanied by two minor UV-detectable impurities. Isolation of the major peak was carried out at a migration time of approximately 13.7 min, which yielded $[M+H]^+$ and $[M+2H]^{2+}$ as the only major ions in the PD mass spectrum (Fig. 2C). Assuming a nearly quantitative efficiency of the hydrostatic sample loading procedure used (see below), a sample amount of approximately 0.02 μg was estimated for the spectrum in Fig. 2C, which is close to the detection limit of PDMS for this polypeptide.

The straightforward applicability of the present CE isolation method, using eqns. 1 and 2 (see Experimental) for determination of transfer times, was demonstrated by PD-MS analyses yielding unequivocal molecular mass identification of several oligo- and polypeptides up to small proteins (see Table I). Relatively large sample amounts (*ca.* 0.1 μg), required to obtain sufficient molecular ion abundances for higher molecular mass polypeptides, can be partly accounted for by using increased hydrostatic loading times. However, even CE separations carried out under capillary overloading conditions did not impede the determina-

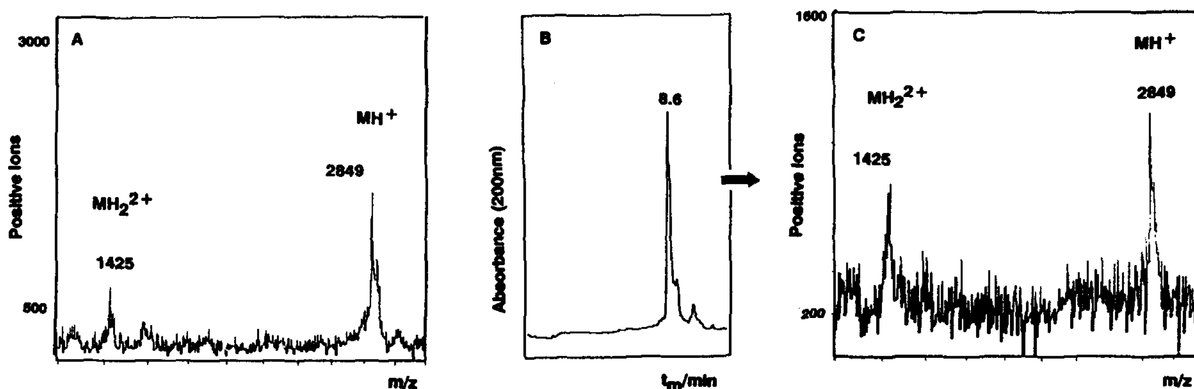

Fig. 2. PD-MS analysis of melittin after CE isolation. (A) PD spectrum (approximately 0.5 μg); (B and C) CE separation and PD spectrum of isolated polypeptide. The estimated sample amount loaded and transferred on to the NC target was 0.2 μg.

tion of suitable isolation conditions for PD-MS analysis.

Transfer efficiency, sensitivity and stability of polypeptides by combined CE–PD-MS analysis

Estimations of the overall sample transfer efficiency and sensitivity of the combined CE–PD-MS analysis were carried out by comparison of molecular ion abundances of model polypeptides under identical PD-MS conditions before and after CE separation, using standard conditions and previously reported yields for hydrostatic sample loading in CE [27]. As structural modification was observed for some polypeptides (see below), the mass spectrometric comparison was considered necessary to obtain reliable quantitative results. PD-MS analyses of 5- and 20-pmol samples of the polypeptides bradykinin and bovine insulin and the corresponding mass spectra after CE isolation and transfer are shown in Fig. 3. Using identical conditions of spectra acquisition (10^7 fission events) for PD-MS [23], comparable intensities of the $[M+H]^+$ ion (m/z 1061) of bradykinin were obtained after hydrostatic loading (60 s) of a 1 nmol/μl sample on to the CE capillary. The PD-MS analysis of insulin gave similar results with molecular ion intensities of good reproducibility in repeated experiments (see legend to Fig. 3). As the loading yields (approximately 5 nl) under these conditions are in good agreement with previously reported values for hydrostatic sample loading in CE [27], this indicates a high efficiency ($\geqslant$ 80%) of the sample isolation and transfer procedure employed. Notable in the PD mass spectra of both polypeptides after CE separation is the observation of an increased mass of molecular ions by 16 u, corresponding to the incorporation of an oxygen atom (see below). In the spectrum of bradykinin an additional molecular ion (m/z 1077) of minor abundance was found; the broad (unresolved) molecular ion peak of insulin with a centroid mass of m/z 5748 also suggests some oxidative modification.

PD-MS data and migration times for a series of isolated polypeptides and proteins are summarized in Table I. Unequivocal molecular mass determinations by protonated molecular ions were consistently obtained with sample amounts of approximately 1–10 pmol for smaller peptides, and could be increased to approximately 100 pmol of isolated sample if required for the characterization of proteins. Although these figures are significantly beyond typical CE sample amounts for the CE of polypeptides, the high transfer efficiency of the present isolation procedure offers the possibility of applying several microanalytical techniques for structural analysis in combination with mass spectrometry, as shown below.

Further, the advantage of using alternative analytical methods, compared with structural analysis by mass spectrometry only, was clearly suggested from PD-MS analyses showing oxidative structural modification of some of the polypeptides investigated (Table I). Molecular ions due to single and/or multiple oxidation were observed for smaller peptides such as LHRH and angiotensin. A larger molecular mass increase was found with increasing molecular mass of proteins such as lysozyme (M_r 14 306). The formation of molecular ions in addition to $[M+H]^+$ by possible cationization with alkali metal ions from buffer constituents or salt contamination was excluded, as the present sample isolation procedure with a large excess of TFA solution strongly favours protonation and appeared to be tolerant towards salt impurities. Oxidation was observed particularly in peptides containing tryptophan residues and thiourea substituents; however, no detailed structural identification was undertaken in this study. Although electrochemical oxidation of peptides appears possible from the CE separation and/or isolation conditions, no structural identification of such products has yet been reported. Systematic studies to identify oxidation sites in peptides, using different buffers for CE separation and conditions of sample isolation, are in progress.

Applications of combined CE–PD-MS in structural studies of polypeptides

The efficiency of the combined CE isolation–PD-MS method was clearly demonstrated by its integration within primary structure studies of polypeptides and proteins, using chemical and enzymatic modification and degradation reactions. An application example to sequence determination is shown in Fig. 4 by the Edman degradation of angiotensin I, in conjunction with PD-MS analysis. A combined Edman–PD-MS sequencing method has recently been developed [13], in which the stepwise phenyl-

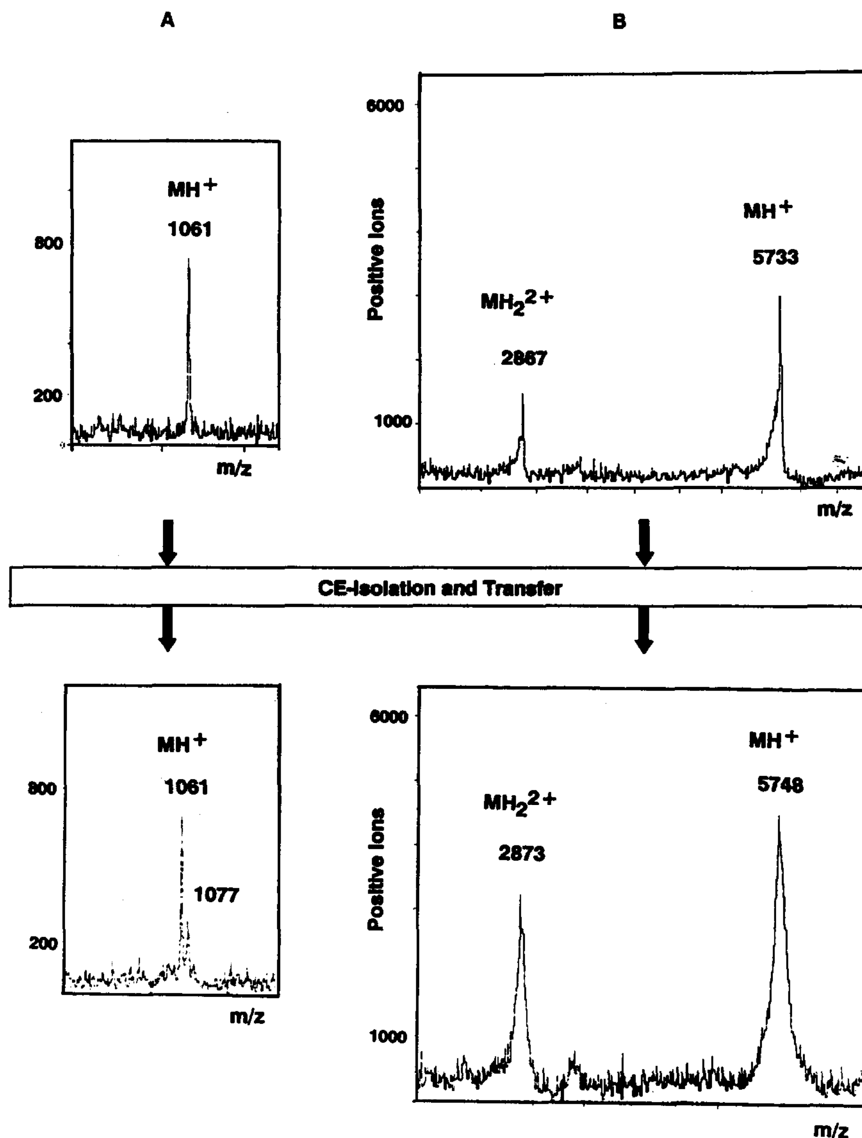

Fig. 3. Determination of sample loading and transfer efficiencies in PD-MS analyses of CE-isolated bradykinin and bovine insulin. (A) PD mass spectrum (molecular ion region) of 5 pmol of bradykinin before CE analysis (upper panel); lower panel, spectrum of CE-isolated peptide obtained from a 1 nmol/μl sample solution by hydrostatic loading for 60 s on to the capillary. (B) PD mass spectra of bovine insulin (0.2 pmol; upper panel) and CE-isolated insulin (lower panel) after loading a solution of 40 pmol/μl.

thiocarbamoyl (PTC) coupling and cleavage products of polypeptides from manual Edman degradation are subjected to PD-MS analysis. The "reverse" sequencing approach by identification of the molecular mass differences of the truncated polypeptide rather than the phenylthiohydantoin (PTH)-amino acid released, has been successfully employed in structural studies, particularly in the simultaneous sequence determination of polypeptide mixtures and modified primary structures [13,28,29].

The PD spectrum of the third phenyl isothiocya-

TABLE I

CHARACTERIZATION OF MODEL PEPTIDES AND PROTEINS BY PD-MS AND CE ISOLATION–PD-MS

Polypeptide		PD-MS		CE isolation–PD-MS			
Sequence	M_r	Sample amount (μg)	$[M+H]^+$ (m/z)	Sample[a] loaded	t_m[b] (min)	$[M+H]^+$ m/z	$[M+H]^+$ Int.[c] (%)
Bradykinin							
H-RPPGFSPFR-OH	1060	0.005	1061	0.005	6.6	1061	100
LHRH							
pEHWSYGLEPG-NH_2[d]	1181	1	1191	–[e]	12.4	1182	60
						1196	40
						1212	100
						1227	20
Neurotensin/pE-LY							
ENKPRRPYIL-OH[d]	1673	1	1674	–[e]	9.4	1673	100
Mellitin/H-GIGAV							
VKVLTTGLPA-							
LISWKRKRQQ-OH	2848	0.5	2849/2878	–[e]	8.6	2849	100
Bovine insulin	5733	0.1	5734	0.1[a]	6.4	5748	100
Hen egg white lysozyme	14 305	1	14 306	–[e]	7.3	14 391[f]	<10
Angiotensin I							
H-DRVYIHPFHL-OH	1297	0.5	1298	0.01	6.0	1298	100
PTC–DRVYIHPFHL-OH[g]	1431	0.5	1432	0.01	6.8	1417	100
						1432	70
						1449	70
PTC–VYIHPFHL-OH[h]	1161	0.5	1162	0.01	10.2	1130	100
						1146	65
						1161	20
H-VYIHPFHL-OH[i]	926	1	927	0.01	6.2	927	100

[a] Sample amount estimated by comparison of $[M+H]^+$ ion abundances in PD-MS and PD-MS after CE separation and isolation.
[b] Migrations time determined by UV detection (*cf.*, Fig. 1).
[c] Relative intensity.
[d] pE, pyroglutamyl.
[e] Not determined.
[f] Molecular mass determined from doubly charged $[M+2H]^{2+}$ ion (*cf.*, Fig. 5).
[g] PTC, phenylthiocarbamoyl; Edman coupling product of angiotensin I.
[h] Coupling product of third Edman degradation cycle of angiotensin I.
[i] Cleavage product of third Edman degradation cycle of angiotensin I.

nate (PITC) coupling step in the Edman degradation of angiotensin I (Fig. 4A) yielded a most abundant $[M+H]^+$ ion at m/z 1161 of the PTC–peptide adduct, PTC–VYIHPFHL, together with a molecular ion resulting from the incomplete preceding cleavage step (m/z 1317; PTC–RVYIHPFHL). The separation of the coupling product mixture by CE afforded a homogeneous major component at a migration time of 10.4 min (Fig. 4B), which was isolated and subjected to PD-MS (Fig. 4C). However, the PD-MS analysis of this peak revealed only a minor $[M+H]^+$ ion due to the intact PTC–peptide. Predominant ions were observed due to molecular mass decreases by 16 and 32 u, respectively, corresponding to oxidative formation of the phenylcarbamoyl–peptide adduct (m/z 1146) and probably further oxidative degradation. Evidence for the oxidative desulphurization of the phenylthiocarbamoyl residue, a well recognized side-reaction of the Edman degradation, was obtained by (i) the finding that the CE-isolated peptide was not amenable to subsequent Edman cleavage; while (ii) direct Edman cleavage of the PTC–peptide afforded molecular ions of the expected product, YIHPFHL, at

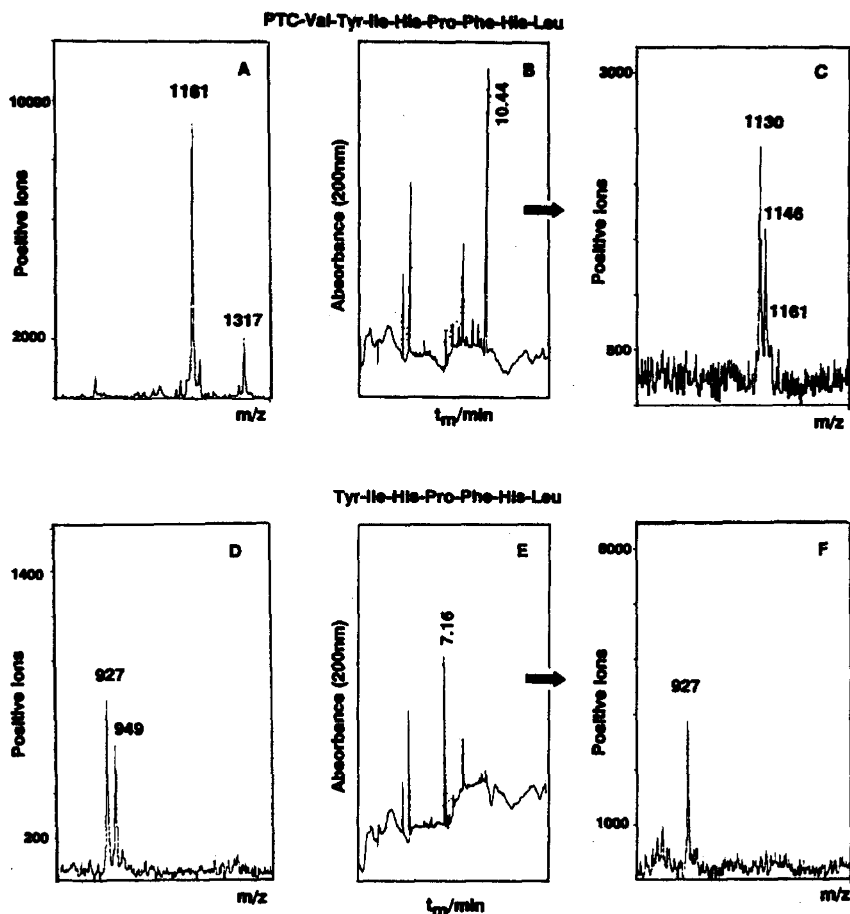

Fig. 4. Identification of PTC coupling and cleavage products from the third Edman degradation cycle of human angiotensin I by PD-MS and CE isolation–PD-MS. (A) $[M+H]^+$ ion of m/z 1161 in PD-MS analysis of PTC–Val–Tyr–Ile–His–Pro–Phe–His–Leu coupling product, showing an additional molecular ion (m/z 1317) from the incomplete preceding degradation step; (B) electropherogram of reaction product mixture from PITC coupling step (A); (C) PD mass spectrum of CE-isolated coupling product with t_m = 10.4 min; (D) PD mass spectrum of TFA cleavage product from the PTC coupling product (A); (E) CE separation of Edman cleavage in (D); (F) PD mass spectrum of CE-isolated peptide from (E) (t_m = 7.16 min).

m/z 927 ($[M+H]^+$) and m/z 949 due to partial formation of the $[M+NA]^+$ ion (Fig. 4G). CE analysis of the TFA cleavage product yielded a major peak at 7.16 min, which showed a homogeneous $[M+H]^+$ ion in the PD mass spectrum (Fig. 4E and F).

Oxidation of PTC–peptides after CE separation and isolation was consistently found in Edman–PD-MS sequence analyses by corresponding molecular ions decreased in mass by 16 u (see Table I). As the formation of the phenylcarbamoyl derivative blocks further Edman degradation, this would preclude purification of PTC coupling products by CE in microsequencing protocols. A procedure obviating this problem is the direct cleavage of PTC adducts and CE isolation of the corresponding cleavage product.

An example of the structural characterization of a protein after CE isolation by direct proteolytic degradation and PD-MS peptide mapping is shown in Fig. 5. Hen egg white lysozyme (HEL) (*ca.* 100 ng), isolated from CE and transferred to PD-MS,

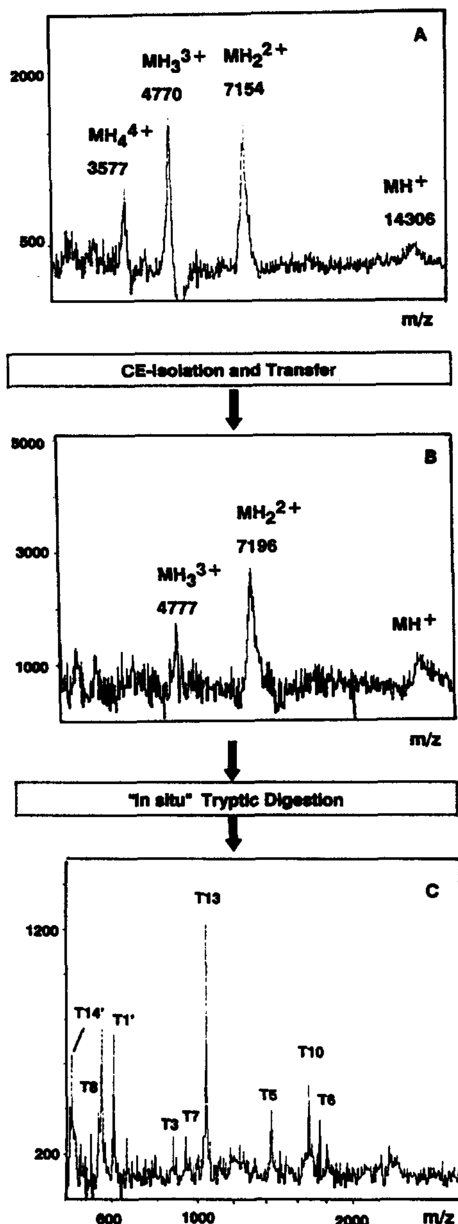

Fig. 5. PD-MS and peptide mapping analysis by *in situ* trypsin digestion of hen egg white lysozyme. (A) PD mass spectrum of HEL (1 μg); (B) PD mass spectrum of HEL after CE isolation and transfer on to the NC target; (C) PD-MS peptide mapping analysis of the HEL sample from (B), after *in situ* DTT reduction and trypsin digestion (see Experimental).

yielded a pattern of singly, doubly and triply charged molecular ions similar to that of the direct PD mass spectrum. However, a significant molecular mass increase by approximately 80 u indicated partial oxidation. The remaining sample from the PD-MS analysis shown in Fig. 5B was subjected to *in situ* DTT reduction and subsequent trypsin digestion on the NC target. In the resulting peptide mapping analysis (Fig. 5C), a series of tryptic peptides were directly identified by their molecular masses expected from the sequence, yielding structural characterization of a substantial part of the protein. The tryptic fragments and partial sequences identified for the CE-isolated protein are compared in Table II with peptide fragments found by direct PD-MS peptide mapping of HEL [12,15]. Notably, no peptides were found for partial sequences (22–33) and (74–96), in which several tryptophan and cysteine residues as possible sites of oxidative modification are located. The present results, however, dit not provide a definite identification of modified structures upon CE separation and isolation, which will be the subject of further studies with the combination of CE and PD-MS.

CONCLUSIONS

An efficient approach for peptide and protein isolation by CE has been combined with PD-MS analysis. Model studies and first applications to polypeptides have shown high efficiency and reproducibility of the CE isolation and transfer procedure and sensitivity of the PD-MS analysis; further, the present approach can be used, in principle, in combination with any CE separation conditions. The combination with PD-MS provides the possibility of applying further analytical methods for the structural characterization of polypeptides, before or subsequent to the mass spectrometric molecular mass determination, *e.g.*, employing *in situ* enzymatic degradation or chemical derivatization reactions [15,29]. Moreover, its application in combination with Edman microsequencing [13] has been demonstrated. In contrast to "on-line" CE–ES-MS methods [30], the off-line CE–PD-MS combination is highly tolerant to buffer salts and different pH conditions, and allows the efficient removal of contaminants from the NC sample target. Therefore, the present approach should prove to be a useful complementary method to "on-line" CE–MS combinations. Also, its evaluation in combination with other desorption–ionization methods such as electrospray and laser desorption [21] should be worthwhile.

TABLE II

COMPARISON OF PD-MS PEPTIDE MAPPING ANALYSIS OF HEL BY DIRECT *IN SITU* TRYPSIN DIGESTION AND HEL AFTER CE ISOLATION

Tryptic peptide fragment	Partial sequence	Calculated M_r	$[M+H]^+$ ions found, m/z	
			Direct PD-MS[a]	PD-MS after CE isolation[b]
T1′	(1–5)	606	607	607
T1	(2–5)	478	478	478
T2	(6–13)	837	–[c]	–[c]
T3	(15–21)	875	875	875
T4	(22–33)	1269	1269	–[d]
T5	(34–45)	1429	1430	1430
T6	(46–61)	1754	1755	1756
T7	(62–68)	937	937	938
T8	(69–73)	517	517	517
T9	(74–96)	2338	2339	–[e]
T10	(98–112)	1676	1677	1677
T11	(113–114)	289	–[f]	–[f]
T12	(115–116)	250	–[f]	–[f]
T13	(117–125)	1046	1046	1047
T14	(126–128)	335	–[f]	–[f]
T14′	(126–129)	448	448	448

[a] Direct *in situ* peptide mapping analysis of HEL [12,15].
[b] *In situ* peptide mapping analysis of CE-isolated HEL.
[c] Not detected owing to molecular ion suppression [12,15].
[d] Peptide containing Trp^{28} and Cys^{30} residues.
[e] Peptide containing Cys^{76}, Cys^{80} and Cys^{96} residues.
[f] Not detected because of background interferences in the low-mass range.

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