ELSEVIER

Journal of Chromatography A, 744 (1996) 177–185

JOURNAL OF CHROMATOGRAPHY A

Comparative peptide mapping of a hepatitis C viral recombinant protein by capillary electrophoresis and matrix-assisted laser desorption time-of-flight mass spectrometry

Martin A. Winkler*, Samar Kundu, Thomas E. Robey, W. Gerard Robey

Diagnostics Division, Abbott Laboratories, North Chicago, IL 60064, USA

Abstract

Capillary electrophoresis (CE) and matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS) were investigated as alternatives to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis for peptide mapping with *Staphylococcus aureus* protease (V8) of a hydrophobic recombinant hepatitis C virus antigen, HC-31, which required 0.1% SDS for solubility. Controls (V8 only) or HC-31 digests were extracted with chloroform–methanol–water (1:4:3) to remove SDS, which interferes with MALDI-TOF, and high salt content, which affects CE. In two different runs by CE, the elution times of each of 11 peptide peaks were very reproducible (R.S.D.<0.016). 25 fragments were resolved by MALDI-TOF-MS, including six smaller peptides (M_r<13 000) resulting from V8 autodigestion. MALDI-TOF-MS indicated that partial cleavages occurred, primarily at sites where there are paired glutamic and/or aspartic acid residues.

Keywords: Peptide mapping; Hepatitis C virus antigen; Peptides; Proteins

1. Introduction

Peptide mapping is a powerful method for characterizing proteins, and has been widely used for checking and validating the lot-to-lot consistency of recombinant and naturally derived proteins. Typically, cleavages by specific proteases such as trypsin are resolved by reversed-phase HPLC [1] or by capillary electrophoresis [2]. Electrospray mass spectrometry coupled with HPLC (LC–MS) can provide accurate identification of proteolytic peptides [3]. More recently, plasma desorption (PD) and matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry have been used for tryptic peptide mapping of recombinant [4,5] or natural [6] proteins. Selective use of a number of different chemical matrices allowed resolution and identification of 96% of the tryptic peptides of recombinant human growth hormone [4]. MALDI-TOF-MS allows rapid identification of peptides, and is more tolerant to buffer constituents and salts than LC–MS. For highly insoluble or very large proteins, digestions for peptide maps may be done in a solution containing sodium dodecyl sulfate (SDS) and endoproteinase Glu-C from *Staphyloccocus aureus* (V8), and the proteolytic fragments resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) by the method of Cleveland [7]. This technique has the advantage of denaturing the protein with SDS to make it more accessible to proteases, and also of solubilizing very hydrophobic or otherwise insoluble proteins. Protein fragments often result which are large enough to be resolved by SDS–PAGE. However, SDS–PAGE has limited capability to resolve smaller proteolytic fragments, is time consuming, and is difficult to quantitate accurately. In this study,

*Corresponding author.

0021-9673/96/$15.00
PII S0021-9673(96)00184-7

we investigate two alternatives to SDS–PAGE for V8 peptide mapping, sieving SDS capillary electrophoresis (CE) and MALDI-TOF-MS. As a model system we chose to peptide map a recombinant hepatitis C protein antigen, HC-31, which requires at least 0.1% SDS to remain soluble. Since MALDI-TOF-MS is inhibited by concentrations of SDS normally used in V8 digestions, we investigated a method of removing SDS from the peptide digest prior to analysis. This method, chloroform–methanol–water extraction [8] was also evaluated as a method of removing salt, which reduces resolution of peptides in CE [9].

2. Experimental

2.1. Proteolytic digestion

HC31 (Abbott Diagnostics, North Chicago, IL) was diluted to 1 mg/ml by the addition of 0.1% SDS in phosphate buffered saline. A 375 μl sample of protein was reduced by addition of 17 μl of 100 m*M* dithiothreitol (DTT) obtained from Pierce. (Rockford, IL, USA) and incubation at 50°C for 15 min. Reduced thiols were alkylated by addition of 37 μl of 100 m*M* iodoacetamide (Sigma-Aldrich, St. Louis, MO, USA) and incubation for 15 min at 22°C. The solution was made 0.1 *M* in dibasic sodium phosphate by addition of a 1 *M* stock solution, then 37.5 μg (1:10, w/w, protease:protein) of *Staphylococcus aureus* protease V8 (Boehringer-Mannheim Biochemicals, Indianapolis, IN, USA) was added, and the samples or controls (V8 in buffer with no HC31) were incubated for 16 h at 22°C. Samples were quick frozen and stored as 100 μl portions at −20°C until analysis. Prior to analysis by CE or MALDI-TOF, 100 μl of digest or control were thawed and SDS and salts removed by the chloroform–methanol–water extraction procedure of Wessel and Flugge [8]). The samples were allowed to air dry for 10 min to remove residual methanol.

2.2. Capillary electrophoresis

Samples (100 μg) or SDS–PAGE molecular mass protein standards-Low (Bio-Rad) were dissolved in 100 μl of CE-SDS sample buffer (0.1 *M* Tris–HCl, pH 9.2 containing 1% SDS) from the CE-SDS protein kit run (Bio-Rad Labs., Hercules, CA, USA) along with internal standard (10 μg benzoic acid), 10 μl DTT (100 m*M*), and 80 μl of water. A 50 μm uncoated capillary (Bio-Rad) was used for all separations performed on a Bio-Rad BioFocus 3000 capillary electrophoresis system, using the software program supplied by the manufacturer for sieving capillary SDS electrophoresis. The capillary was purged for 90 s with 0.1 *M* NaOH and 60 s with 0.1 *M* HCl before filling with CE-SDS Running Buffer (0.4 *M* Tris–borate, pH 8.3 containing 0.1% SDS and Bio-Rad proprietary sieving polymer). Samples were injected electrophoretically for 5 s at 10 kV. Running time was 15 min at 15 kV at room temperature. Absorbance was monitored at 220 nm. Molecular masses were calculated using the SDS-Gel CE worksheet for Microsoft Excel (Hewlett-Packard, Naperville, IL, USA).

2.3. MALDI-TOF-MS

Dried samples (100 μg) were resuspended in 50 μl of 0.1% trifluoroacetic acid (TFA, Pierce) and 5 μl samples were diluted two or five-fold in 0.1% TFA and 5 μl of the dilutions mixed with 5 μl of sinapinic acid matrix (Hewlett-Packard, Protein Chemistry Systems, Palo Alto, CA, USA), which is 100 m*M* sinapinic acid in 0.1% TFA, 25% methanol, and 25% acetonitrile. A Hewlett-Packard G2025A MALDI-TOF linear system was used for analysis. The instrument was previously calibrated on the same day with bovine serum albumin, cytochrome *c*, and myoglobin protein standards (Hewlett-Packard). At least 50 laser shots at 4 μJ per shot were accumulated for each sample and the best spectra were obtained with the 5-fold dilutions of sample.

2.4. Protein-peptide analysis software

The Peptide Tools software package for mass spectrometry (Hewlett-Packard) and Procomp (Dr. P.C. Andrews, University of Michigan, Ann Arbor, MI, USA) were used to predict proteolytic fragment mass from the HC-31 amino acid sequence (K. Rupprecht, Abbott Laboratories, North Chicago, IL, USA).

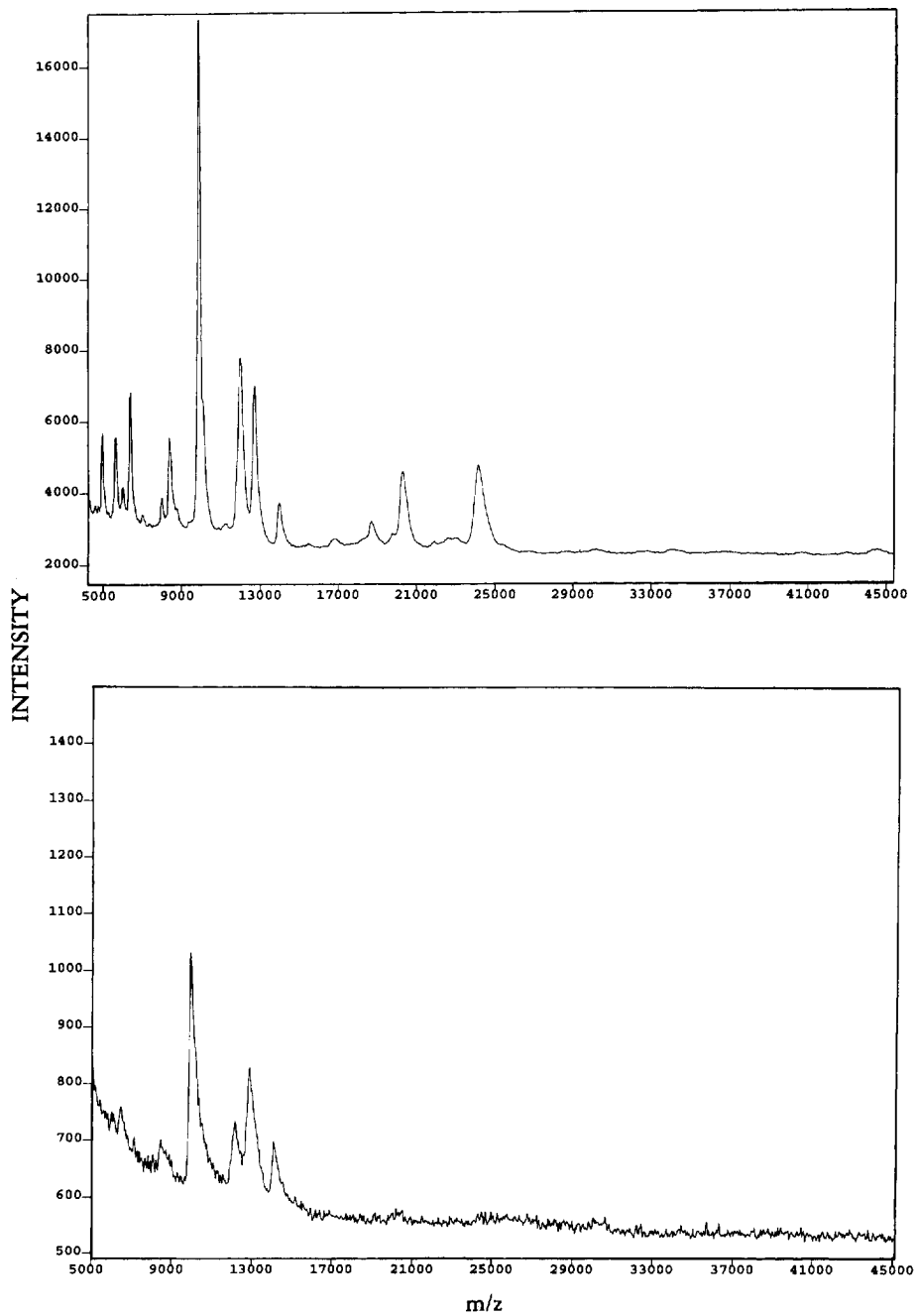

Fig. 1. MALDI-TOF mass spectrometry of a chloroform–methanol–water extract of a V8 digest (1:10, w/w protease:protein) of 100 μg reduced and alkylated HC-31. Samples were dissolved in 0.1% trifluoroacetic acid and diluted five-fold before mixing with sinapinic acid matrix (100 mM) in 1% TFA–acetonitrile–methanol (2:1:1). The mass/charge ratio (m/z) is shown versus the relative intensity for the sum of 50 laser shots.

3. Results

MALDI-TOF mass spectra of a sample of the peptide digests are shown in Fig. 1 along with the V8 control. At least 25 different peptides were resolved in the HC-31 V8 digest (Fig. 1 top and Fig. 2) and ranged in size from 4959 to 24 203 relative molecular mass units. The control V8 protease digest showed 6 peptides (Fig. 1 lower), due to autodigestion, at 6446, 8448, 9953, 12 169, 12 898, and 14 127 rel. mol. mass units. Table 1 shows that these peptides are close to cleavages predicted from the amino acid sequence of V8 [10]. Four of these products of autodigestion appear to be present in the V8 protease digest of HC31 (Fig. 1 top) at 6384, 9885, 12 749, and 14 017 rel. mol. mass units, though authentic HC31 peptides may also be at or near these masses. Because of the peptides resulting from V8 autodigestion, the MALDI-TOF peptide map of HC31 was selected as 15 000 rel. mol. mass through 26 000 rel. mol. mass. The peptide map in this range is shown in Fig. 2. Table 1 shows the masses of the V8 and HC31 peptides determined by MALDI-TOF. Computer software was used to select peptides resulting from specific V8 cleavages which were closest in mass to the observed peptide masses. Only one peptide, V8 92-219, came within the calibrated accuracy of the instrument (<0.1%). None of the other peptide masses was close enough (<0.1% of predicted) to assign them conclusively to a predicted proteolytic fragment and no further studies were done to confirm their identity. Therefore these assignments should be considered the most likely ones but are tentative. The lack of accuracy of the observed peptide masses compared with the protein mass spectrometry standards may be due to the presence of residual SDS at an average of 2 mols or less. Alkylation of reduced sulfhydryls may also have been sterically hindered by SDS bound near the cysteines. Three peptides, HC31 135–331, HC 567–781 and HC 550–781, appear to have 1–2 mols SDS remaining, assuming that the assignments are correct. Most fragments in the peptide map appear to correspond to predicted V8 cleavage products of HC-31 at

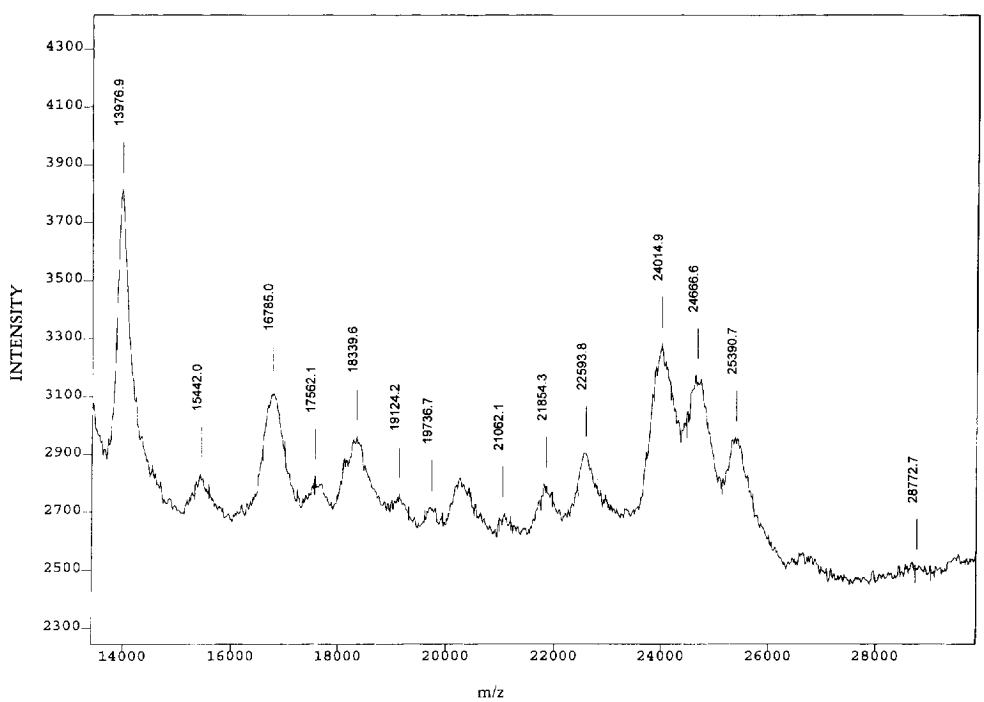

Fig. 2. Peptide map of the V8 digest HC-31 from the preparation shown in Fig. 1. The peptide at 21 062 was not labeled by the instrument software though it is significant in size.

Table 1
MALDI-TOF-MS of V8 and HC-31 peptides

Peptide	Found M_r	Predicted M_r	Difference (rel. mol. mass)	% Difference
V8 219–76	6446	6361	85	1.30
V8 201–276	8448	8509	61	0.72
V8 123–212	9958	9907	51	0.51
V8 104–219	12 898	12 747	192	1.18
V8 111–219	12 169	11 940	229	1.92
V8 92–219	14 127	14 134	7	0.05
HC-31 135–258	13 976	13 996	20	0.14
HC-31 135–268	15 442	15 262	50	1.17
HC-31 90–238	16 785	16 775	13	0.08
HC-31 373–550	19 124	19 063	61	0.30
HC-31 548–750	21 062	20 996	66	0.31
HC-31 135–331	21 854	21 508	346	1.60
HC-31 567–781	22 593	22 291	302	1.40
HC-31 550–781	24 014	24 309	295	1.20
HC-31 547–781	24 666	24 699	33	0.13
HC-31 101–331	25 390	25 318	72	0.28

the carboxyl terminal of glutamic acid or aspartic acid residues (Table 1). Of the 42 glutamic acid residues and 36 aspartic acids, only 17 of these appear to be cleaved (Fig. 3). Cleavages appear to be more likely at paired glutamic, aspartic, or Asp–Glu residues. Table 2 shows that in two peptide maps, the run-to-run R.S.D. values for the mass determinations on two different days were less than 0.0064 (mean= 0.002). Two peptide maps of the same portion run on different days are shown in Fig. 4. The pattern and

```
        10         20         30         40         50         60         70
MSFVVIIPAR YASTRLPGKP LVDINGKPMI VHVLERARES GAERIIVATD HEDVARAVEA AGGEVCMTRA

        80         90        100        110        120        130        140
DHQSGTERLA EVVEKCAFSD DTVIVNVQGD EPMIPATIIR QVADNLAQRQ VGMTTLAVPI HNAEEAFNPN

       150        160        170        180        190        200        210
AVKVVLDAEG YALYFSRATI PWDRDRFAEG LETVGDNFLR HLGIYGYRAG FIRRYVNWQP SPLEHIEMLE

       220        230        240        250        260        270        280
QLRVLWYGEK IHVAVAQEVP GTGVDTPEDL DPSTNSMAVD FIPVENLETT MRSPVFTDNS SPPVVPQSFQ

       290        300        310        320        330        340        350
VAHLHAPTGS GKSTKVPAAY AAQGYKVLVL NPSVAATLGF GAYMSKAHGI DPNIRTGVRT ITTGSPITYS

       360        370        380        390        400        410        420
TYGKFLADGG CSGGAYDIII CDECHSTDAT SILGIGTVLD QAETAGARLV VLATATPPGS VTVPHPNIEE

       430        440        450        460        470        480        490
VALSTTGEIP FYGKAIPLEV IKGGRHLIFC HSKKKCDELA AKLVALGINA VAYYRGLDVS VIPTSGDVVV

       500        510        520        530        540        550        560
VATDALMTGY TGDFDSVIDC NTCNSSTGCV VIVGRVVLSG KPAIIPDREV LYREFDEMEE CSQHLPYIEQ

       570        580        590        600        610        620        630
GMMLAEQFKQ KALGLLQTAS RQAEVIAPAV QTNWQKLETF WAKHMWNFIS GIQYLAGLST LPGNPAIASL

       640        650        660        670        680        690        700
MAFTAAVTSP LTTSQTLLFN ILGGWVAAQL AAPGAATAFV GAGLAGAAIG SVGLGKVLID ILAGYGAGVA

       710        720        730        740        750        760        770
GALVAFKIMS GEVPSTEDLV NLLPAILSPG ALVVGVVCAA ILRRHVGPGE GAVQWMNRLI AFASRGNHVS

       780
PWDPLDCRHA K
```

Fig. 3. Amino acid sequence of pHCV-31 (K. Rupprecht, Abbott Laboratories, Abbott Park, IL, USA). The methionine at position one is removed in most expressed protein. Aspartic and glutamic acid residues most likely to be cleaved in the V8 digests are underlined and in bold.

Table 2
MALDI-TOF-MS of HC-31 peptide map: run-to-run comparison

Peptide no.	M_r first run	M_r second run	S.D.	R.S.D. (%)
1	15442.0	15437.3	2.4	0.02
2	16785.0	16763.2	10.9	0.06
3	17562.1	17339.7	111.2	0.64
4	18399.6	18277.9	60.9	0.33
5	19124.2	19054.4	34.9	0.18
6	19736.7	19610.7	63.0	0.32
7	20267.1	20235.8	15.7	0.08
8	21854.3	21752.8	50.8	0.23
9	22593.8	22531.4	31.2	0.14
10	24014.9	23931.0	42.0	0.17
11	24666.6	24597.4	34.6	0.14
12	25390.7	25320.0	35.3	0.14

masses are very similar except for reduced yield for the masses at 24 014, 24 666 and 25 390 rel. mol. mass on the second day.

SDS-sieving CE of the V8 digest of HC-31 run on three different days of samples of the same HC-31 digest are shown in Fig. 5 (top trace: day 1; middle trace: day 2; bottom trace: day 3). 11 fragments were resolved, and they ranged in size from 9400 to 23 700 rel. mol. mass units. Peaks of absorbance were also seen for the internal standard (benzoic acid) at 3.2 min and DTT at 9.6 min. Only these two peaks were seen in the V8 control digests and blank runs (data not shown). The relative absorbance on day 3 was one-fourth of the other two days, and may be due to less recovery of peptides during the methanol–chloroform extraction, since the internal standard was undiminished. However, the R.S.D.s of the relative elution times of each peak of absorbance in the first two days (Table 3) were less than 0.016 (average=0.0094), showing that the separation method is highly reproducible. The SDS–PAGE gels of the same digests (data not shown) showed four major bands from 9000 to 25 000 rel. mol. mass units, four minor bands in this region, and two bands corresponding to V8 protease at 42 000 rel. mol. mass for a total of ten bands. No V8 autodigestion fragments were detected by SDS–PAGE. For two of the CE runs, the sensitivity of peptide detection (11 peaks of absorbance) exceeded that of the coomassie stained SDS gels. No autodigestion peptides and no intact V8 were detected in the V8 control, in contrast to the results for MALDI-TOF. Since V8 autodigestion

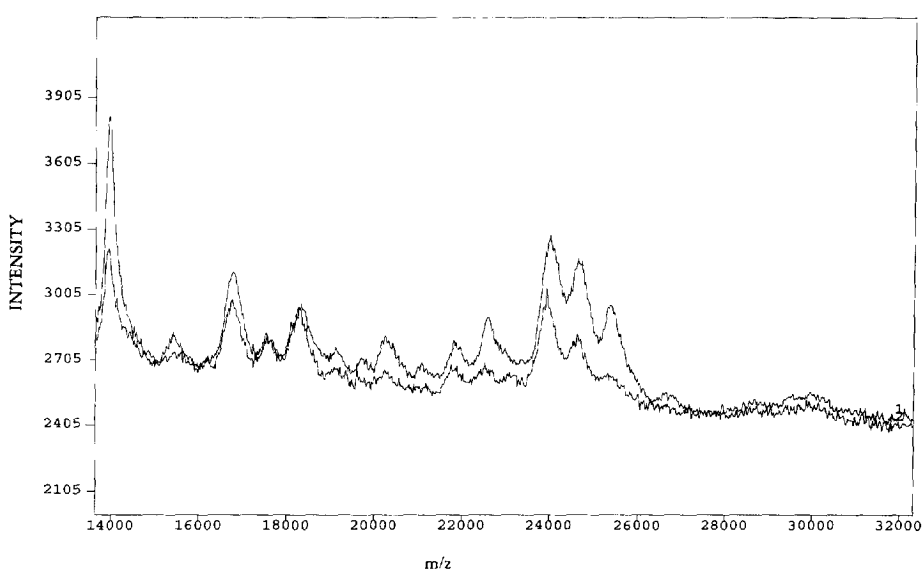

Fig. 4. Comparison of two peptide maps of HC-31 run on MALDI-TOF on two different days. The digest was prepared as described in the legend to Fig. 1. The top tracing is the first run, the bottom is the second.

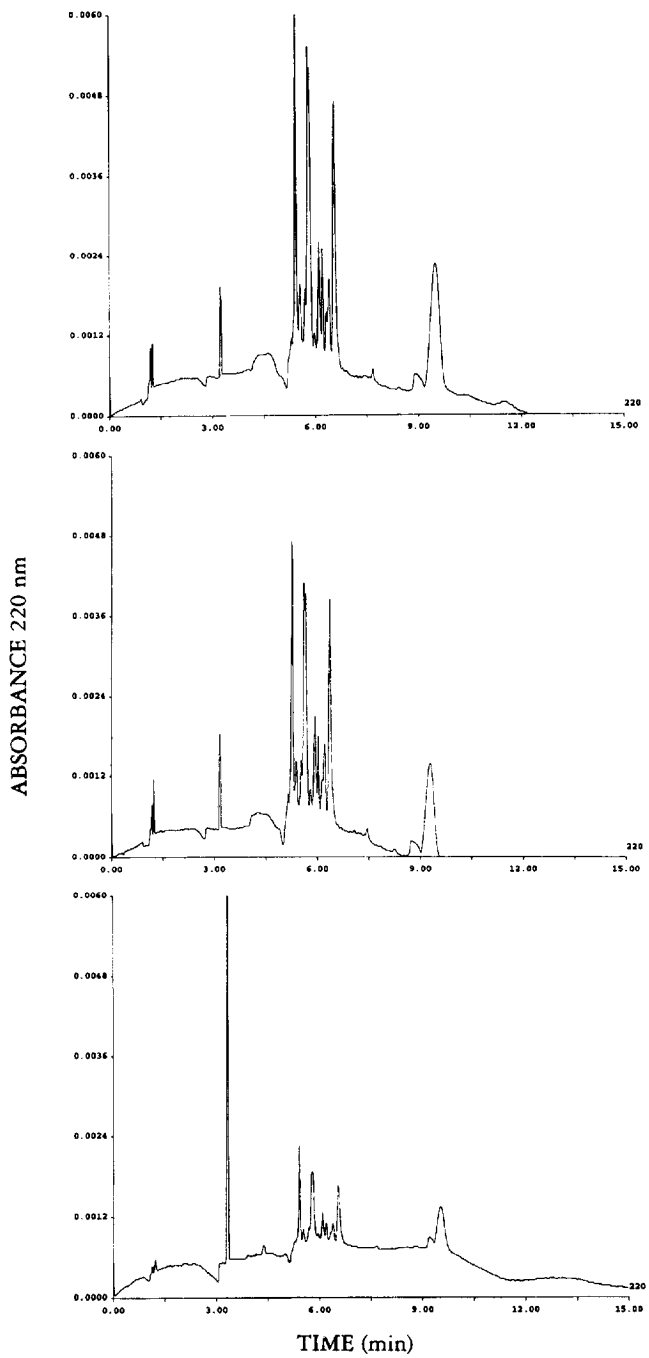

Fig. 5. Sieving capillary electrophoresis of a V8 digest of reduced an alkylated HC-31. The digest was prepared as described in the legend to Fig. 1. The dried extract of (100 μg) was mixed with 10 μg benzoic acid (internal standard) and DTT to 5 m*M*, boiled for 10 min in CE-SDS sample buffer and analyzed in the CE-SDS running buffer (0.4 *M* Tris–borate, pH 8.3 containing 0.1% SDS and Bio-Rad sieving polymer) by the program supplied with the BioFocus 3000 CE. Top trace: CE on day 1 of first chloroform–methanol–water extract. Middle trace: CE on day 2 of second chloroform–methanol–water extract. Bottom trace: CE on day 3 of third chloroform–methanol–water extract.

Table 3
Capillary electrophoresis of HC-31 V8 fragments

Peptide no.	Relative migration time		Calculated molecular mass		S.D. mol. mass	R.S.D. (%) mig. times
	First run	Second run	First run	Second run		
1	0.6065	0.6113	9417	9020	198.5	0.39
2	0.5918	0.5886	10720	11035	157.5	0.27
3	0.5779	0.5748	12128	12469	170.5	0.27
4	0.5686	0.5606	13166	14132	17.0	0.71
5	0.5636	0.5519	13765	15269	752.0	1.05
6	0.5509	0.5472	15398	15922	262.0	0.34
7	0.5389	0.5230	17140	19732	1296.0	1.50
8	0.5299	0.5137	18554	21420	1438.0	1.55
9	0.5195	0.5055	20337	23021	1342.0	1.37
10	0.5137	0.4992	21420	24349	1464.5	1.43
11	0.5024	0.4878	23680	26948	1634.0	1.47

products were detected by mass spectrometry, the CE apparently did not have the sensitivity to detect these peptides.

4. Discussion

The CE sieving separations were highly reproducible, with run-to-run variation less than 1.6% for all proteolytic fragments. The mass determinations by MALDI-TOF were also highly reproducible run-to-run, with R.S.D.s less than 0.007. MALDI-TOF resolved 25 proteolytic fragments of HC-31, with 19 due to specific cleavage of HC-31 and 6 due to V8 autodigestion. Eleven proteolytic fragments were resolved by CE and none of these appeared to be due to autodigestion of V8. Ten fragments were resolved by SDS–PAGE. Smaller peptides (M_r<9000) were not resolved by CE. Therefore CE has limitations similar to SDS–PAGE in resolving small proteolytic fragments and did not have the sensitivity to detect V8 autodigestion products. In contrast, the MALDI-TOF spectral peak patterns were not as reproducible as the CE patterns. Three fragments of higher mass (>24 000 rel. mol. mass) showed variable yields in intensity in the MALDI-TOF analysis. Since the same samples were analyzed by both CE and MALDI-TOF, and the MALDI-TOF instrument is calibrated to an accuracy of less than 0.1%, the variation in MALDI-TOF is probably not due to variation between the samples or variation in the efficiency of the instrument. One possible source of variation is the matrix crystallization with the protein, which could vary from run to run. Another is residual quantities of SDS, which did not appear to be completely removed by the extraction procedure. Billeci and Stults [4] and Tsarbopoulos [5] have shown that detection and resolution of tryptic peptides by MALDI-TOF required selective use of several different chemical matrices. Future work with other matrices, such as they describe, may increase the reproducibility of the peptide maps. Overall, CE seems most appropriate for lot-to-lot validation of proteins where a reproducible pattern is required. MALDI-TOF can supply more detailed information about fragment masses and possible cleavages giving rise to them, but did not show the same overall pattern run-to-run. Therefore MALDI-TOF may be sensitive to structural variations between different purified lots of protein, but does not allow one to guarantee, at the present time, reproducible peptide maps of the protein lot-to-lot.

One challenge of this work was to identify a method of removing high salt (necessary during the V8 digest) and SDS (necessary to keep HC-31 soluble). The chloroform–methanol–water extraction of Wessel and Flugge was used to accomplish both goals. Since no useful MS spectra were obtained unless this procedure was used, it appears to remove most of the SDS. For some peptides, especially three with molecular masses over 21 000, residual SDS with an average between one and two mols appeared to remain. Capillary electrophoresis required the extraction procedure for resolution of fragments. The

extraction procedure appears not to affect the reproducibility of the CE peptide map pattern, though yields of the peptides were seen to be diminished in one of the three runs by CE. Because this extraction method is rapid, adaptable to micro scale, and simple to perform, it may be useful to other CE or MALDI-TOF applications requiring removal of salts and de-bulking of SDS.

Acknowledgments

We thank Dr. Tim Wehr of Bio-Rad, Hercules, CA, USA for training and assistance with the capillary electrophoresis, and Ms. Lin Costagli of Hewlett-Packard, Naperville, IL, USA, for Peptide Tools and CE molecular mass software.

References

[1] H. Chen and C. Horvath, J. Chromatogr. A, 705 (1995) 3.
[2] R.M. McCormick, in J.P. Landers (Editor), Handbook of Capillary Electrophoresis, CRC Press, Boca Raton, FL, 1994, pp. 287.
[3] J.R. Yates III, S. Speicher, P.R. Griffin and M.W. Hunkapiller, Anal. Biochem., 214 (1993) 397.
[4] T.M. Billeci and J.T. Stults, Anal. Chem., 65 (1993) 1709.
[5] A. Tsarbopoulos, M. Karas, K. Strupat, B.N. Pramanik, T.L. Nagabhushan and F. Hillenkamp, Anal. Chem., (1994) 2062.
[6] M. Schar, K.O. Bornsen and E. Gassman, Rapid Comm. Mass Spectrom., 5 (1991) 319.
[7] D.W. Cleveland, S.G. Fisher, M.W. Kirschner and U.K. Laemmli, J. Biol. Chem., 252 (1977) 1102.
[8] D. Wessel and U.I. Flugge, Anal. Biochem., 138 (1984) 141.
[9] R.P. Oda and L.P. Landers, in J.P. Landers (Editor), Handbook of Capillary Electrophoresis, CRC Press, Boca Raton FL, 1994, p. 9.
[10] S. Hua, M.M. Vestling, C.M. Murphy, D.K. Bryant, J.J. Height, C. Fenselau, J. Theibert and J.H. Collins, Int. J. Peptide Prot. Res., 40 (1992) 546.