ELSEVIER

Journal of Chromatography A, 867 (2000) 151–160

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Peptide map procedure using immobilized protease cartridges in tandem for disulfide linkage identification of *neu* differentiation factor epidermal growth factor domain

Shinichi Hara*, Viswanatham Katta, Hsieng S. Lu

Amgen Inc., Dept. of Protein Structure, M/S 14-2-E, Thousand Oaks, CA 91320, USA

Received 27 May 1999; received in revised form 11 October 1999; accepted 14 October 1999

Abstract

Immobilized proteolytic enzyme cartridges were used to rapidly digest *neu* differentiation factor EGF domain in order to obtain improved peptide maps useful for assignment of disulfide linkages. The procedure described here involves an on-line digestion of proteins using immobilized trypsin and endoproteinase Glu-C cartridges connected in series, followed by on-line RP-HPLC separation of the peptides. The entire process can be automated using a commercially available workstation; and the total time required for both proteolytic digestion and the HPLC separation can be shortened to within 1 h. Using these immobilized columns, we demonstrated that disulfide structure assignment of the EGF domains of recombinant human *neu* differentiation factor can be performed by isolation of individual disulfide-containing peptides followed by assignment of disulfide linkages with prompt fragmentation of peptides using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The use of immobilized protease cartridges in tandem eliminates undesirable digestion artifacts associated with longer digestion time and higher protease-to-substrate ratio and results in the development of a reproducible and high quality peptide map. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Peptide mapping; Immobilized enzymes; *Neu* differentiation factor; Disulfide linkages; Peptides

1. Introduction

Formation of a biologically functional molecule with correct disulfide linkages by in vitro folding process is critical when disulfide-containing protein molecules are expressed in a bacterial expression system. Peptide mapping is frequently employed to evaluate primary structure of the isolated proteins and to confirm correctness of the disulfide structure [1,2]. However, obtaining a useful and reliable peptide map may demand optimization of conditions for both proteolytic digestion and HPLC analysis. For proteins containing multiple disulfide bonds, the digestion processes may require the use of a high enzyme-to-substrate ratio in combination with longer incubation time and elevated temperature. Such digestion conditions inevitably create digestion artifacts such as nonspecific cleavage [3–6], methionine oxidation [7,8], disulfide mispairing [9,10], transpeptidation [6,11], or deamidation [12–14] of the substrates as well as autodigestion of the proteolytic enzyme [15]. We have experienced such problems in

*Corresponding author. Tel.: +1-805-447-4654; fax: +1-805-499-7464.

E-mail address: shara@amgen.com (S. Hara)

0021-9673/00/$ – see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0021-9673(99)01110-3

the disulfide mapping of *neu* differentiation factor (NDF*) [16] and IGF-I molecules [6,10]. For example, disulfide structure determination of the NDF-EGF domain using a combination of endoproteinase Lys-C and Glu-C digestion in solution took an extensive incubation time for a more complete cleavage [16].

Proteolytic enzymes immobilized on solid support have been demonstrated to enhance cleavage efficiency so that the digestion time can be dramatically reduced [17–19]. As demonstrated here, digestion of NDF-EGF domain with trypsin and Glu-C immobilized cartridges was completed in 5 min at room temperature. The digestion can be combined with on-line RP-HPLC for separation of the peptides, which shortened the analysis time required for digestion and separation to approximately 1 h. The eluted peaks can be rapidly analyzed by off-line matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) for confirmation of disulfide linkages. The method described here is potentially applicable to develop an improved peptide mapping method useful for the determination of disulfide structure of proteins with less quantity of sample amount. Moreover, the rapid analysis in both digestion and HPLC has substantially improved peptide map quality by increasing cleavage efficiency with better recovery and minimizing generation of cleavage artifacts as described.

2. Materials and methods

2.1. Materials

NDF-EGF-α2 and -β1 molecules were purified according to the published method [16]. Tris-(hydroxymethyl)aminomethane (Tris) was purchased from Sigma (St. Louis, MO, USA) and $CaCl_2$ was from J.T. Baker (Phillisburg, NJ, USA). Acetonitrile was purchased from EM Science (Gibbstown, NJ, USA) and Burdick & Jackson (Muskegon, MI, USA). Sequencing grade trypsin and endoproteinase Glu-C were obtained from Boehringer Mannheim, Germany.

2.2. In-solution trypsin and Glu-C digestion and peptide mapping

Samples were digested in solution to compare the recovery of on-line digested peptides. Fifty μg of samples were dried by SpeedVac and reconstituted in 20 m*M* Tris, pH 8.0 to make a final concentration of 1 mg/ml. One μg of trypsin was added to the sample to give enzyme-to-substrate ratio of 1:50. The sample was digested for 15 h at 37°C. Then 2 μg of Glu-C enzyme was added to the trypsin-digested sample and further incubated for an additional 10 h at 37°C. The digestion was quenched with a final 0.5% concentration of trifluoroacetic acid (TFA) and stored at −20°C. Two, three, five and ten μg of digested peptides were analyzed on a HP 1090 liquid chromatograph (Hewlett-Packard, Mountain View, CA, USA) by Zorbax 300SB-C_{18} RP column (150×2.1 mm, Mac-Mod Analytical, Chadds Ford, PA, USA). Peptides were eluted at a linear gradient of 10–50% buffer B for 50 min at a flow-rate of 0.15 ml/min, followed by 90% buffer B wash for 15 min. Solvents used to elute peptides were 0.05% TFA in water (buffer A) and acetonitrile–0.045% TFA in water (90:10) (buffer B). All RP-HPLC was performed at an ambient temperature throughout this study.

2.3. Automated on-line digestion and peptide mapping

On-line digestion and peptide mapping was performed on an automated Integral Micro-Analytical Workstation (PerSeptive Biosystems, Framingham, MA, USA). The instrument was set according to the manufacturer's protocol. Approximately 20 μg of protein samples were automatically injected into the workstation with a built-in autosampler and passed at room temperature slowly through two immobilized enzyme cartridges, Poroszyme Trypsin and Glu-C columns (30×2.1 mm, PerSeptive Biosystems). Enzymes were covalently crosslinked to poly(styrene–divinylbenzene) beads under proprietary methods according to PerSeptive Biosystems. Each column contained approximately 5 mg immobilized protease concentration. Two columns were connected in a series where the protein was digested into peptide

fragments. The digestion buffer was according to the manufacturer's protocol, i.e., 50 m*M* Tris, pH 8.0 with 10 m*M* $CaCl_2$ flowing at a rate of 50 μl/min allowing approximately a 5-min digestion in each of the 100 μl immobilized enzyme cartridges. As the peptide fragments eluted from the immobilized enzyme cartridges, they were directed onto an RP column for capture and subsequent analysis. A volume of 0.5 ml of digestion buffer was used to transfer the peptides to the RP column after which the workstation was automatically purged with the solvents used for RP analysis. Solvents used to elute peptides were 0.1% TFA in water (buffer A) and acetonitrile–0.1% TFA in water (80:20) (buffer B). Peptide fragments were eluted with a linear gradient of 5–70% buffer B for 30 min, using PepMap C_{18} RP column (100×4.6 mm, PerSeptive Biosystems) at a flow-rate of 1 ml/min. Collected fractions were analyzed by MALDI-TOF-MS and N-terminal protein sequencer.

2.4. Manual on-line digestion and peptide mapping

Manual on-line digestion was developed in such a configuration that Poroszyme trypsin and Glu-C cartridge columns (30×2.1 mm) were connected in tandem prior to Zorbax 300 SB-C_{18} RP column (150×2.1 mm) on a HP 1090 liquid chromatograph. One to five μg of protein samples were injected into immobilized enzyme cartridges and the protein in the cartridges was digested by passing 50 m*M* Tris, pH 8 with and without 10 m*M* $CaCl_2$ at a flow-rate of 20–50 μl/min for 30 min at room temperature. The immobilized cartridges were removed from the LC system when a 30-min digestion run was finished, and the RP column was directly reconnected on the LC system. The RP column was then washed for 20 min at a flow-rate of 0.15 ml/min with 100% buffer A (0.05% TFA in water) to remove excess volume of digestion buffer and to equilibrate the column. Finally, the digested peptides were eluted at a linear gradient of 0% buffer B (acetonitrile–0.045% TFA in water, 90:10) to 50% buffer B for 50 min at a flow-rate of 0.15 ml/min, followed by 90% buffer B wash for 15 min at room temperature. The eluted peptides were manually collected and subjected to Edman sequence analysis and/or mass spectrometric analysis.

2.5. MALDI-TOF-MS analysis

The collected fractions were analyzed using a MALDI-TOF mass spectrometer (Voyager DERP, PerSeptive Biosystems). The instrument was operated in linear mode (20 kV acceleration, 92% Grid, 0.1% guidewire, 500 MHz digitizer) and employed delayed extraction (100 ns delay) for improving resolution. The m/z scale was externally calibrated using a mixture of known peptide samples on a different spot. Typically, 1–10 pmol of the sample (0.5–1.0 μl) is mixed with an equal volume of matrix solution on the sample plate. 4-hydroxy-α-cyano cinnamic acid was prepared in acetonitrile–0.1% TFA solution (1:1, v/v). Spectra signal arising from 50 to 100 laser shots (nitrogen laser, 337 nm wavelength, <3 ns pulse width) were averaged. The data were smoothed (5-point) and analyzed using GRAMMS/386 software.

2.6. N-Terminal sequence analysis

The peptide sequence was determined by HP G1000A Protein Sequencer (Hewlett-Packard) and ABI Model 477 automated sequencer (Applied Biosystems, Foster City, CA, USA) using sequencing programs recommended by the manufacturers.

3. Results and discussion

3.1. In solution digestion

As shown in Fig. 1, both NDF-EGF-α2 and -β1 contain 65 and 70 amino acids, respectively, and each molecule has three intramolecular disulfide bonds. The expected peptide fragments containing single disulfide bond of the α2 and β1 isoforms can be obtained by proteolytic digestion with a combination of trypsin and Glu-C. Attempts with this approach have been made to optimize the in-solution digestion conditions to obtain better peptide maps for disulfide bond assignment of NDF-EGF-α2 and -β1 isoforms. Tris buffer (pH 8.0 in the range of 10–50

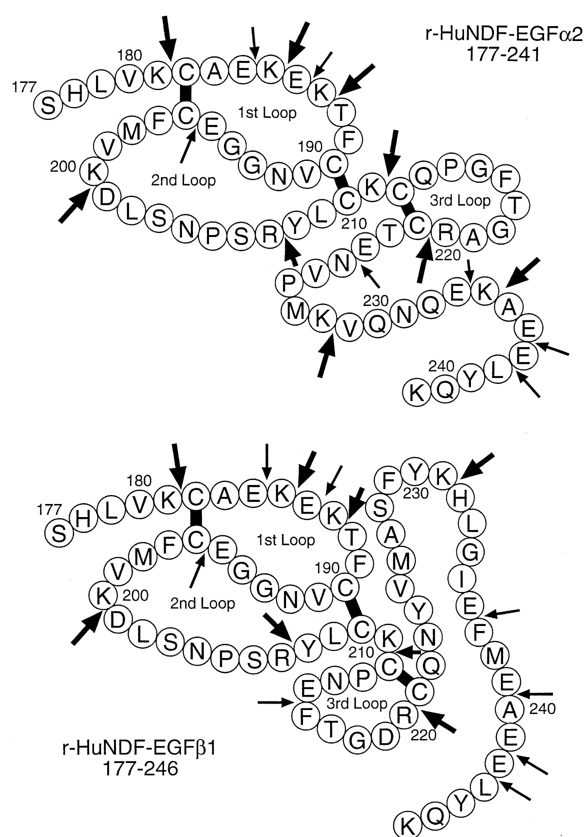

Fig. 1. Covalent disulfide structure of recombinant human NDF-EGF-α2 and -β1 isoforms. Expected cleavage sites for trypsin and Glu-C are indicated as thick and thin arrows, respectively.

m*M*) with or without 10 m*M* $CaCl_2$ was found to generate a maximal number of peptides. The use of other buffers such as NH_4HCO_3 (pH 7.8) or sodium phosphate (pH 7.2) actually deteriorates generation of NDF peptides. Fig. 2(A) and (B) show typical peptide maps for in-solution digestion of the α2 and β1 isoforms with 5-μg load by both trypsin and Glu-C. Sequence analysis of peptides found in labeled peaks were performed and summarized in Table 1. As shown in Fig. 2, hydrophilic peptides, i.e., peaks a, 1, 2 and b, were eluted close to the injection peak, but the gradient developed here was sufficient enough to separate their peptides for identification. HPLC maps for both isoforms showed great column reproducibility in retention time for those peptides identical to both α2 and β1 isoforms.

Peak 5 from both isoforms contains three peptides connected by two disulfide bonds, which was observed previously that glutamate residue at 195 was not effectively cleaved by Glu-C enzyme [16]. Peptides of peak 3 and peak 4 were generated from peak 5 upon cleavage of Glu^{195} by Glu-C. Peak 4 from both isoforms contains two peptides connected by a single disulfide, Cys^{190}–Cys^{210}. Characterization of peak d shown in Fig. 2A confirms that it is identical to peak 4 and contains Asn^{192} being deamidated to isoaspartate residue for NDF-EGF-α2. This peptide contains an Asn–Gly sequence, which has a high propensity for deamidation in vitro [12–14]. It is interesting to note that no deamidated peptide corresponding to peak 5 of NDF-EGF-α2 (Fig. 2(A)) was observed, suggesting that deamidation of Asn^{192} may be associated with generation of peptide 4 after cleavage of Glu^{195} in NDF-EGF-α2. In contrast, a deamidated peptide h derived from peptide 5 was observed in the β1 isoform but not found in the α2 isoform (see Fig. 2(B)). The deamidation appears to occur during in-solution digestion as the original sample preparation has been evaluated to contain very little deamidated species [16].

In-solution digestion of the α2 isoform appears to generate a better map for further analysis, whereas in-solution digestion of the β1 isoform generates a more complicated map (Fig. 2(B) and Table 1). It was also observed that peptide bonds located near the disulfide loops are not completely digested, thereby a number of disulfide-containing peptides with various peptide lengths are generated.

It should be noted that trypsin digestion of both isoforms has to take place prior to subfragmentation by Glu-C. We observed that digestion is relatively incomplete when the digestion order was reversed. Similar results were also observed during on-line digestion of the α2 isoform when immobilized enzyme cartridges were connected in series first with Glu-C cartridge and second with trypsin cartridge. Since both enzyme cartridges contain approximately 5 mg of immobilized enzymes as described in the method section, the enzyme-to-substrate ratio is probably not critical. It appears that specific bond cleavage by trypsin is more crucial to develop a complete peptide map for NDF-EGF protein. In reverse digestion condition as described above, elution of tryptic fragments for Glu-C enzyme leaching

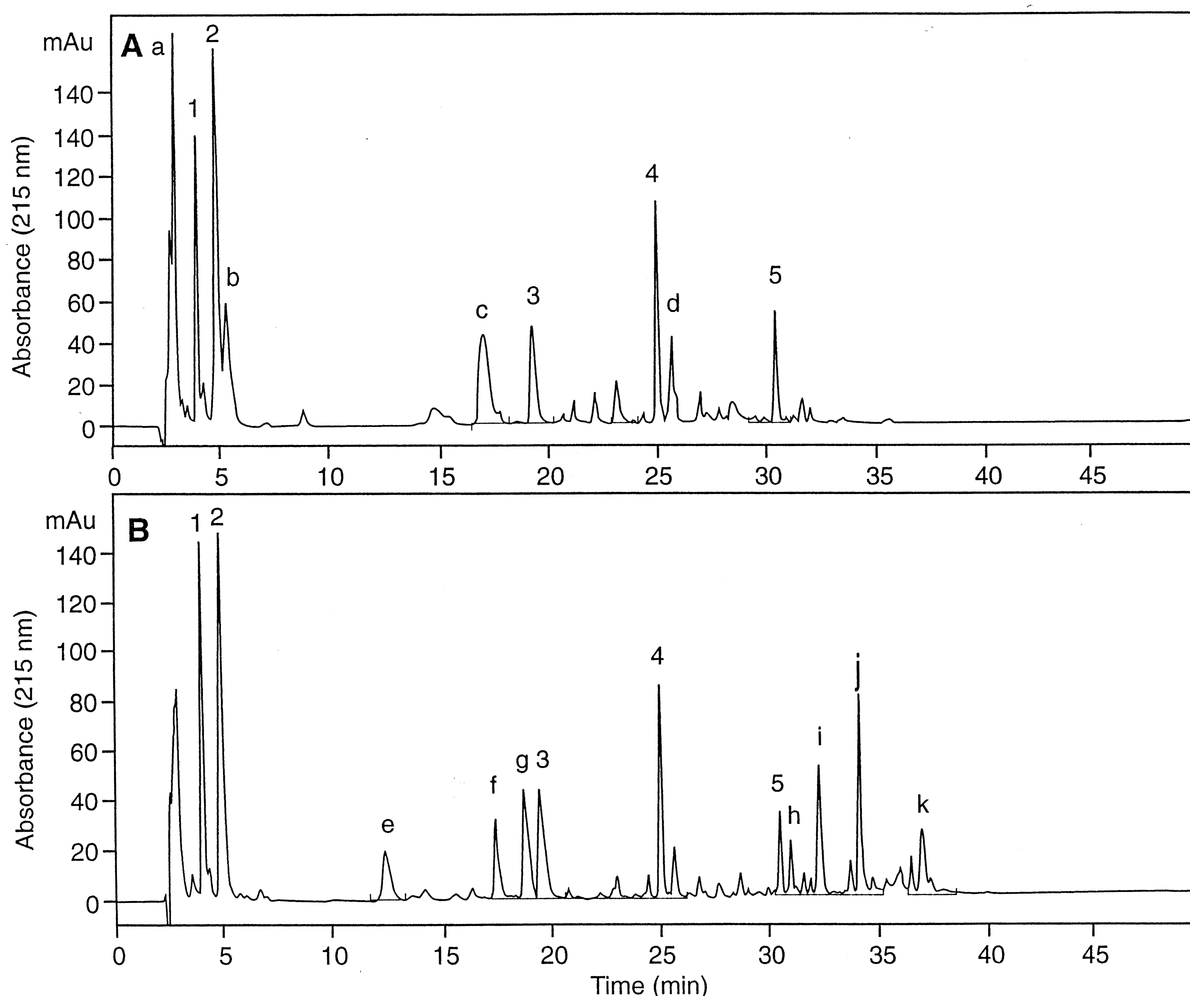

Fig. 2. In-solution digestion and peptide mapping of the α2 isoform (A) and the β1 isoform (B). Elution of peptides with 10–50% buffer B for 50 min followed by 90% buffer B for 15 min. Peak identification is listed in Table 1.

from the immobilized column was also observed at a significant level, which could potentially interfere with the interpretation of a peptide map.

In-solution digestion also generates a number of smaller peaks, due to incomplete digestion of NDF isoforms and autolysis of proteases. Different digestion conditions using a higher amount of protease with a shorter digestion time, addition of organic solvent (e.g., 10% acetonitrile) and detergent (2–3 *M* urea) and/or digestion at a lower pH have been evaluated. $CaCl_2$ (10 m*M*) was also included in the digestion buffer to eliminate autolysis and incomplete digestion. However, no apparent improvement was observed with these attempts (data not shown here). As a conclusion, in-solution digestion of NDF-EGF domains with trypsin and Glu-C resulted in the generation of incomplete cleavage of protein substrates, in situ generation of deamidated peptides, and autodigestion of proteolytic enzymes. These artifacts have limited the use of peptide map for further characterization, especially for confirming correct structure of disulfide linkages. Thus, it is obvious that in-solution digestion of NDF-EGF domains may not be the best choice. This has led us

Table 1
Analysis of peptides obtained from in-solution digest

Peptides	EGF-α2		EGF-β1		Disulfide linkage
	Sequence	Position	Sequence	Position	
1	LYQK	(238–241)	LYQK	(243–246)	
			FTGDR	(214–220)	
2	SHLVK	(177–181)	SHLVK	(177–181)	
	DLSNPSR	(201–207)	DLSNPSR	(201–207)	
3	CAEK	(182–185)	CAEK	(182–185)	Cys^{182}, Cys^{196}
	CFMVK	(196–200)	CFMVK	(196–200)	
4	TFCVNGGE	(188–195)	TFCVNGGE	(188–195)	Cys^{190}, Cys^{210}
	YLCK	(208–211)	YLCK	(208–211)	
5[a]	CAEK	(182–185)	CAEK	(182–185)	
	TFCVNGGECFMVK	(188–200)	TFCVNGGECFMVK	(188–200)	
	YLCK	(208–211)	YLCK	(208–211)	
a	VQNQEK	(229–234)			
b	NVPMK	(224–228)			
c	CQPGFTGAR	(212–220)			Cys^{212}, Cys^{221}
	CTE	(221–223)			
d	TFCisoDGGE	(188–195)			
	YLCK	(208–211)			
e			FME	(237–239)	
f[a]			FMEAEE	(237–242)	
g			HLGIE	(232–236)	
h[a]			CAEK	(182–185)	
			TFCVisoDGGECFMVK	(188–200)	
			YLCK	(208–211)	
i			CPNE	(212–215)	Cys^{212}, Cys^{221}
			CQNYVMASFYK	(221–231)	
j[a]			CPNEFTGDR	(212–220)	
			CQNYVMASFYK	(221–231)	
k[a]			TFCVNGGE	(188–195)	
			YLCKCPNEFTGDRCQNYVMASFYK	(208–231)	

[a] Peaks were found to contain peptides incompletely digested by Glu-C.

to evaluate alternative digestions including the use of immobilized proteolytic enzyme cartridges.

3.2. Peptide mapping using immobilized protease cartridges in tandem

An initial experiment using on-line digestion with immobilized protease cartridges was carried out at ambient temperature. Fig. 3(A) and (B) show the peptide maps generated by automated on-line digestion and mapping of the α2 and β1 isoforms, respectively. Improvement in the peak recovery and reduction of undesired peaks was also observed. As indicated in Table 2, both α2 and β1 isoforms generated five identical peaks (i.e., peaks 1–5). Peaks a and b from the α2 isoform (Fig. 3(A)) and peaks c and d from the β1 isoform (Fig. 3(B)) are unique fragments. Peak b was confirmed to have two sequences of CQPGFTGAR and CTE linked by a disulfide. Mass spectrometric analysis of peak b as shown in Fig. 4(A) indicated that molecular mass of peptide is 1286.31, matching the theoretical monoisotopic MH+ of being disulfide-linked peptide (1286.55 u). Fig. 4(A) also shows a prompt fragment for the peptides generated in situ during MALDI-MS analysis. Sequence analysis of peak 4 revealed two sequences of CAEK and CFMVK with recovery of PTH-cystine at the first sequence cycle, suggesting that cystines of the peptides are linked by an S–S bond. Fig. 4(B) shows the mass spectrometric analysis of peak 4; and the observed MH+ of 1075.15 u matches the theoretical monoisotopic MH+ of the

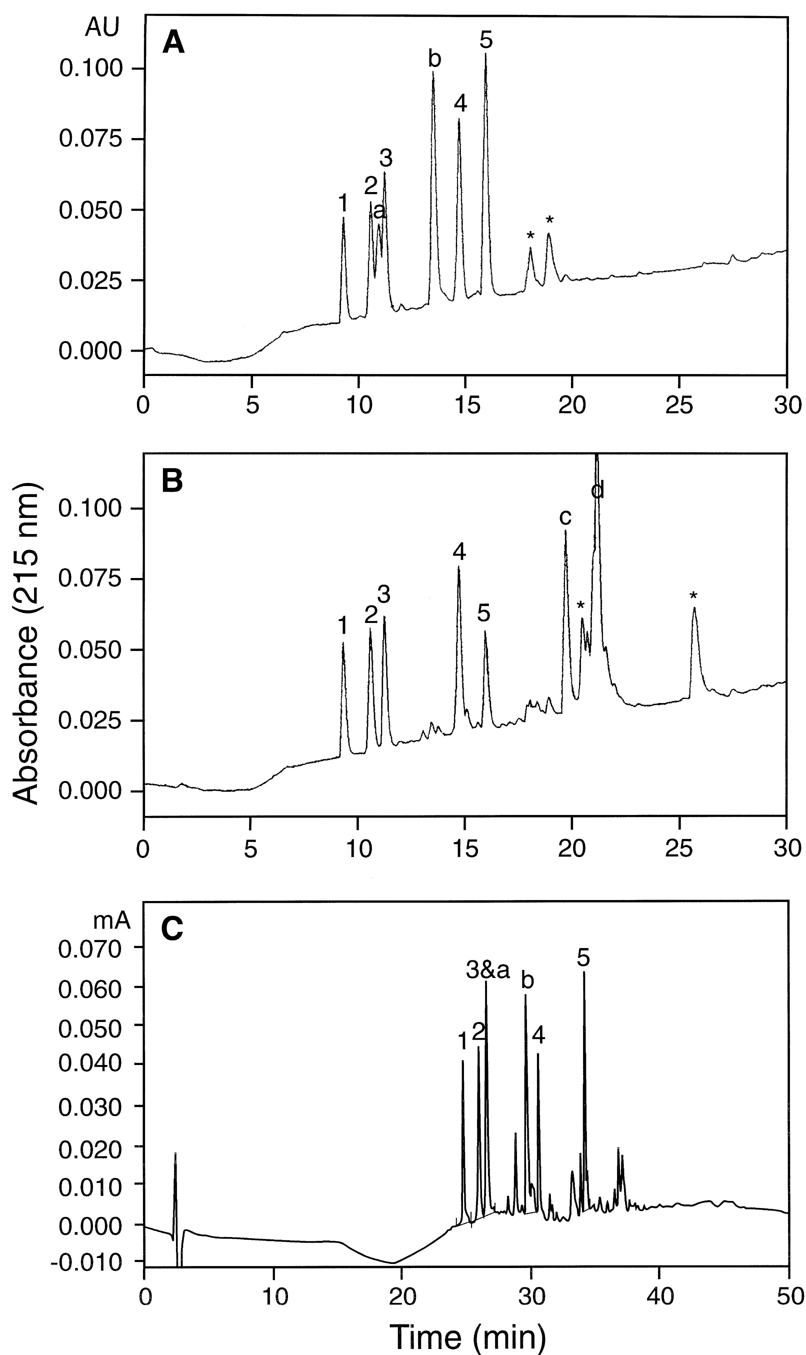

Fig. 3. Automated on-line digestion and peptide mapping of the α2 isoform (A) and the β1 isoform (B). Elution of peptides with 5–70% buffer B for 30 min. Peaks labeled as asterisks contained either incompletely digested peptides or intact protein. Manual on-line digestion and peptide mapping of the α2 isoform (C). Elution of peptides with 0–50% buffer B for 50 min followed by 90% buffer B for 10 min. For details, see Section 2. Peak identification is listed in Table 2.

Table 2
Analysis of peptides obtained from poroszyme trypsin and Glu-C cartridges

Peptides	EGF-α2		EGF-β1		Disulfide linkage
	Sequence	Position	Sequence	Position	
1	LYQK	(238–241)	LYQK	(243–246)	
2	SHLVK	(177–181)	SHLVK	(177–181)	
3	DLSNPSR	(201–207)	DLSNPSR	(201–207)	
4	CAEK	(182–185)	CAEK	(182–185)	Cys^{182}, Cys^{196}
	CFMVK	(196–200)	CFMVK	(196–200)	
5	TFCVNGGE	(188–195)	TFCVNGGE	(188–195)	Cys^{190}, Cys^{210}
	YLCK	(208–211)	YLCK	(208–211)	
a	NVPMK	(224–228)			
b	CQPGFTGAR	(212–220)			Cys^{212}, Cys^{221}
	CTE	(221–223)			
c[a]			CPNEFTGDR	(212–220)	Cys^{212}, Cys^{221}
			CQNYVMASFYK	(221–231)	
d[a]			HLGIEFMEAEE	(232–242)	

[a] Peaks were found to contain peptides incompletely digested by Glu-C.

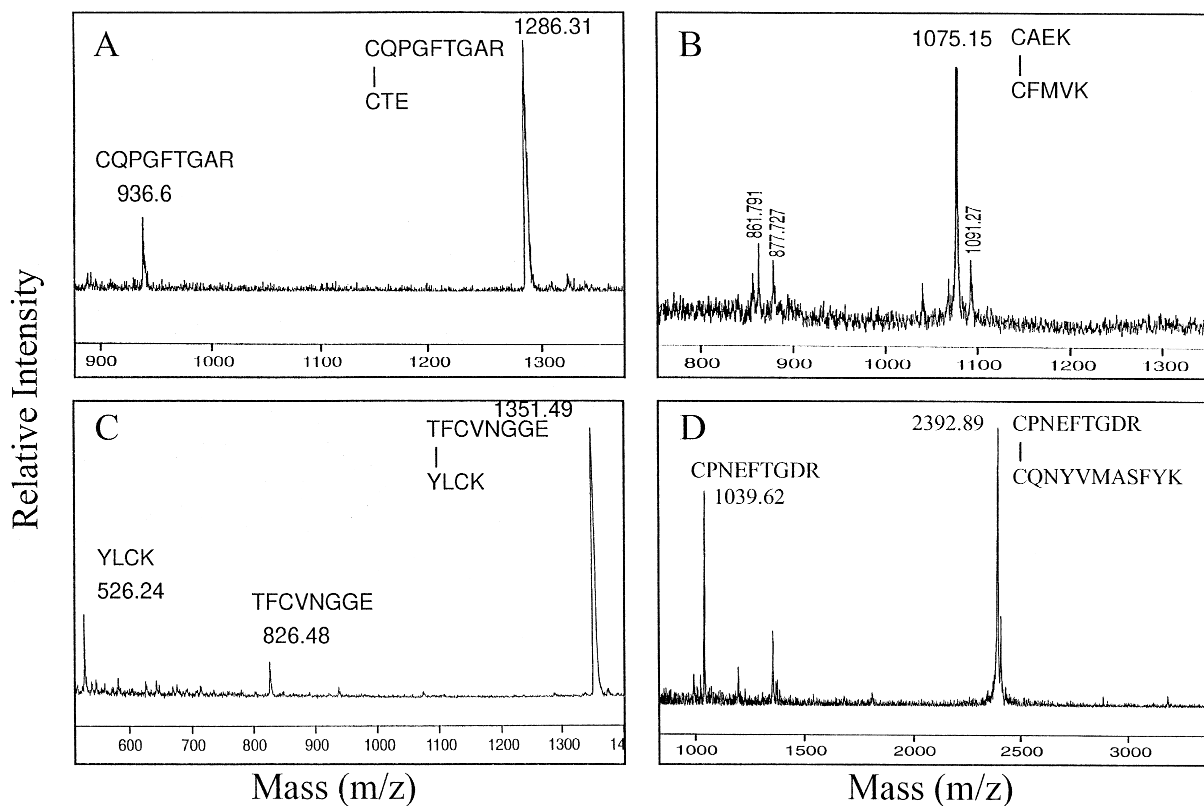

Fig. 4. MALDI-TOF-MS analysis of disulfide peptides for the α2 and the β1 isoforms. (A) Peak b containing the third disulfide bond in the α2 isoform. (B) Peak 4 containing the first disulfide bond in the α2 and β1 isoforms. (C) Peak 5 containing the second disulfide bond in both isoforms. (D) Peak c containing third disulfide bond in the β1 isoform.

disulfide-linked peptide (1075.50 u). The observed *m/z* 1091.27 appears to be a methionine-oxidized form, presumably generated during sample preparation for mass spectrometry. From these results, a disulfide-linked peptide containing CAEK and CFMVK sequence in the first disulfide loop region can be separated from a peptide in the second disulfide loop region. Sequence analysis of peak 5 revealed two sequences of TFCVNGGE and YLCK in the second loop region. The molecular mass of 1351.0 for the peptide (Fig. 4(C)) matches the theoretical monoisotopic MH+ for the disulfide-linked peptide of TFCVNGGE and YLCK (1350.61 u). Thus, the expected disulfide structure of the α2 isoform can be readily identified using MALDI-TOF-MS analysis of the isolated disulfide-linked peptides.

Fig. 3(B) shows peptide map of the β1 isoform by automated on-line digestion and mapping. As indicated, disulfide-linked peptides (peaks 4 and 5) corresponding to the first and second disulfide loops are identical to those of the α2 isoform peptides. The disulfide-linked peptide in the third disulfide loop was eluted as peak c with sequences of CPNEFTGDR and CQNYVMASFYK. The mass spectrometric analysis of the peptide showed molecular mass of 2392.89 (Fig. 4(D)), matching the theoretical average MH+ of 2391.03 u. Sequence analysis of peak d (Fig. 3(B)) revealed a single sequence of HLGIEFMEAEE without a disulfide bond (see Table 2).

Fig. 3(C) shows a typical map of the α2 isoform from a manual on-line digestion in 50 m*M* Tris, pH 8.0 using Poroszyme trypsin and Glu-C cartridges in series. Absorbance at 230 nm was monitored to detect no elution of peptides during the digestion and equilibration flows. During these initial flows, absorbance at 230 nm instead of 215 nm was taken due to a Tris buffer interference below 230 nm. It was also observed to be critical that no acetonitrile buffer should be mixed with aqueous buffer during equilibration of the column. When a small percentage of acetonitrile buffer was present, peak 1 and/or peak 2 were lost in the equilibration flow or in the injection peak of a peptide mapping run. Peaks indicated in the Fig. 3(C) were also identified and listed in Table 2. We found that addition of 10 m*M* $CaCl_2$ during digestion yielded a similar peptide map. On-line digestion for the α2 isoform in either automated fashion or manual fashion gave an identical profile as shown in Fig. 3(A) and (C).

It was also observed that a significant amount of the β1 isoform was left uncleaved or incompletely digested (asterisks in Fig. 3(B)) while digestion of the α2 isoform was nearly complete (Fig. 3(A)). When the β1 isoform digest was performed at a flow-rate of 20 μl/min, these peaks could be minimized (data not shown). However, a complete digestion of the β1 isoform has never been achieved even at a lower flow-rate or at higher digestion temperature, i.e., 35°C. The result suggests that the difference in the C-terminal sequence may generate a different conformation that affects the cleavage efficiency by protease. Inaccessibility of the β1 isoform to the immobilized Glu-C enzyme was evidenced by the observation that cleavages at Glu 215, 236, 239 and 241 in the β1 isoform did not occur. A similar observation was made when both isoforms were digested in solution by endoproteinase Lys-C [16]. We observed that Lys^{211} for the β1 isoform was less susceptible for a cleavage than the α isoform despite the identical sequence in the N-terminal region. Cleavage at Lys^{211} by Lys-C can be overcome by continuously adding more Lys-C enzyme during digestion (data not shown).

When less than 1 μg of the α2 isoform was directly digested by manual on-line method, other peaks previously not seen in the 2 μg load became significant (data not shown). Although not all of the peaks were analyzed, sequence analysis of selected peaks revealed that at least some are Glu-C fragments, derived from tryptic fragmentation of the Glu-C protein. We speculate that a very small amount of leached trypsin, which flowed into the second cartridge, is responsible for the cleavage of the Glu-C protein. These phenomena may be inherent for the immobilized enzyme column. Thus, these leached proteins or peptides may be constantly present at a less significant level, but may become prominent during high sensitivity analysis. In the present on-line digestion conditions, NDF samples at 2 μg load appear to be the limit without significant interference of peptides leached out of the protease cartridges.

In general, trypsin and Glu-C immobilized columns are stable and can be repeatedly used without

losing proteolytic activity. Thus multiple usage for sample digestion can be performed with a relatively stable baseline. Traces of trypsin and Glu-C fragments are present and may potentially be interfered with peptide separation during high-sensitivity micro-peptide mapping.

4. Conclusion

The use of immobilized enzyme cartridges in tandem has significantly shortened the time required for proteolytic digestion. The procedures generate peptide digests with significantly less nonspecific cleavage or other modifications such as in situ deamidation and oxidation. HPLC mapping of the peptide digests can be achieved either by an automatic integrating system or by a manual on-line mode. A complete digestion using cartridges in series would generate disulfide-containing peptides ready for rapid assignment of disulfide bonds using MALDI-TOF-MS following prompt fragmentation. It should be noted that in-solution digestion at higher pH could generate mispaired disulfide-linkage as a result of disulfide shuffling. It has been previously observed that Lys-C and Glu-C digestion of NDF-EGF domain detected minor species containing mispaired disulfide-bonds [16]. However, no peptide containing mispaired disulfide-bonds was observed in the peptide mapping generated by on-line digestion. Thus the method described here may be useful in characterizing the structure of protein molecules and the determination of disulfide linkages, when the in-solution digestion procedure would not generate desirable peptide digest for subsequent structural characterization.

5. Abbreviations

EGF	Epidermal growth factor
IGF-I	Insulin growth factor-I
LC	Liquid chromatography
MALDI-TOF-MS	Matrix-assisted laser desorption ionization time of flight mass spectrometry
NDF	*Neu* differentiation factor
RP	Reversed-phase
TFA	Trifluoroacetic acid

Acknowledgements

The authors with to thank Ms. Joan Bennett for manuscript preparation and Mr. Doug Paulin for figure illustration. The authors also thank Mr. Ken Miller and Dr. Tim Nadley from PerSeptive Biosystems, Inc. for an opportunity to use the Integral Workstation and for helpful discussion with this manuscript.

References

[1] C.S. Fullmer, R.H. Wasserman, J. Biol. Chem. 254 (1979) 7208–7212.
[2] W.J. Kohr, R. Keck, R.N. Harkins, Anal. Biochem. 122 (1982) 348–359.
[3] G. Allen, in: T.S. Work, R.H. Burdon (Eds.), Laboratory Techniques in Biochemistry and Molecular Biology, North-Holland, New York, 1981, pp. 58–59.
[4] G.L. Francis, K.A. McNeil, J.C. Wallace, F.J. Ballard, P.C. Owens, Endocrinology 124 (1989) 1173–1183.
[5] J.E. Layton, G. Morstyn, L.J. Fabri, G.E. Reid, A.W. Burgess, R.J. Simpson, E.C. Nice, J. Biol. Chem. 266 (1991) 23815–23823.
[6] S. Hara, R. Rosenfeld, H.S. Lu, Anal. Biochem. 243 (1996) 74–79.
[7] N. Brot, H. Weissbach, Trends Biochem. Sci. 7 (1982) 137–139.
[8] V.R. Anecetti, B.A. Keyt, W.S. Hancock, Trends Biotechnol. 7 (1989) 342–348.
[9] B.N. Violand, J. Tou, B.D. Vineyard, N.R. Siegel, C.E. Smith, P.D. Pyla, J.F. Zobel, P.C. Toren, E.W. Kolodziej, Int. J. Peptide Protein Res. 37 (1991) 463–467.
[10] R.D. Rosenfeld, N.M. Noone, S.L. Lauren, M.F. Rohde, L.O. Narhi, T. Arakawa, J. Protein Chem. 12 (1993) 247–254.
[11] E. Canova-Davis, T.J. Kessler, V.T. Ling, Anal. Biochem. 196 (1992) 39–45.
[12] A.B. Robinson, C.J. Rudd, Curr. Top. Cell Regul. 8 (1974) 247–295.
[13] S. Clarke, Int. J. Peptide Res. 30 (1987) 808–821.
[14] R. Bischoff, H.V.J. Kolbe, J. Chromatogr. B 662 (1994) 261–278.
[15] P.C. Andrews, J.E. Dixon, Methods Enzymol. 168 (1989) 97.
[16] S. Hara, N. Liu, S.-Y. Meng, H.S. Lu, Biochim. Biophys. Acta 1292 (1996) 168–176.
[17] S. Malekni, J.D. Dixon, J. Mark, N.B. Afeyan, Presented at the 42nd ASMS Conference on Mass Spectrometry and Allied Topics, Chicago, IL, 1994, abstract.
[18] D.B. Kassel, T.G. Consler, M. Shlaby, P. Sekhri, N. Gordon, T. Nadler, Techn. Protein Chem. VI (1995) 39–46.
[19] F. Hsieh, H. Wang, C. Elicone, J. Mark, S. Martin, F. Regnier, Anal. Chem. 68 (1996) 455–462.