Short-Chain Peptide Analysis by High-Performance Liquid Chromatography Coupled to Electrospray Ionization Mass Spectrometer after Derivatization with 9-Fluorenylmethyl Chloroformate

Karin Gartenmann and Sunil Kochhar*

Nestlé Research Center, P.O. Box 44, Vers-chez-les-Blanc, CH-1000 Lausanne 26, Switzerland

Resolution and characterization of short-chain peptides (M_r = 200−1000) and free amino acids were demonstrated by the use of precolumn derivatization with 9-fluorenylmethyl chloroformate (Fmoc) followed by reverse-phase high-performance liquid chromatography (RP-HPLC) interfaced with an electrospray ionization mass spectrometer (ESI-MS). At pH 10, in addition to derivatization at the N terminus, ϵ-NH_2 and OH groups of lysine and tyrosine residues, respectively, were also derivatized. Fmoc derivatives showed at least 2 orders of magnitude higher ionization potential in the presence of trifluoroacetic acid. The detection levels for both the free amino acid and peptide derivatives were in a few hundred picomoles compared to 10−50 nmol for the underivatized samples. The mass spectra of the peptides before or after derivatization showed the presence of only singly charged ions. However, collision-induced dissociation of the derivatized peptides showed predominance of *b*-type ions that are relatively less complicated in assigning the peptide sequence.

Keywords: *Amino acids; ESI-MS; Fmoc; HPLC/MS; peptides; short-chain peptides*

INTRODUCTION

Maillard reactions between amine groups and sugars are responsible for color and flavor generation in foods. A number of reaction products and their sensory attributes have been documented for Maillard chemistry of amino acids and sugars [see Izzo and Ho (1992) and references cited therein]. Short-chain (SC) peptides play a significant role in flavor and aroma formation, and there is a growing interest to better understand the Maillard chemistry of peptides. However, an important prerequisite is the isolation, purification, and characterization of naturally occurring peptides in a given food system, for example, coffee, cocoa, meat, or plant protein hydrolysates. Typically, following purification, Edman degradation sequencing, combined with amino acid analysis, characterizes a peptide. Benchtop electrospray ionization mass spectrometry interfaced to liquid chromatography allows simultaneous detection of coeluting molecules and circumvents the need for a high degree of purification. However, detection in a low molecular mass range (M_r = 50−400) is rather insensitive due to relatively high chemical noise level and poor ionization of peptides and amino acids. Recently, Cárdenas et al. (1997) reported an on-line derivatization of peptides with *N*-succinimidyl-2(3-pyridyl)acetate to improve microcapillary reverse-phase high-performance liquid chromatography/liquid chromatography/mass spectrometry (RP-HPLC/LC/MS) detection at the femtomole level. However, the reagent is unstable and laborious to synthesize. Furthermore, the methodology addressed identification and characterization of medium-chain peptides (7−15 amino acid residue).

* Author to whom correspondence should be addressed (telephone +41 21 7859336; fax +41 21 7858925; e-mail sunil.kochhar@rdls.nestle.com).

9-Fluorenylmethyl chloroformate (Fmoc), a protective reagent for amino groups during peptide synthesis, has been shown to be highly suitable for fluorescence labeling of primary and secondary amines (Anson Moye and Boning, 1979). Over the years it has emerged as a useful reagent for sensitive amino acid analysis following precolumn derivatization (Einarasson et al., 1983). The reaction conditions, quantitative yields, and stability of derivatives have been carefully established (Einarasson et al., 1983). Fmoc derivatization has been used for the purification of hydrophilic peptides in dairy products (Roturier et al., 1995). In this paper, we present data on LC/MS detection of amino acids and peptides at picomole levels after precolumn derivatization with the Fmoc reagent.

EXPERIPENTAL PROCEDURES

Materials. Synthetic peptides (2−9 amino acid residues) were obtained from Bachem, Switzerland. The peptide selection criteria were their hydrophilic/hydrophobic properties and chain length. Stock solutions of peptides (0.1 M) were prepared in 50% (v/v) acetic acid and stored at −20 °C. HPLC grade acetonitrile (ACN) and methanol, trifluoroacetic acid (TFA), 9-fluorenylmethyloxy carbonyl chloride (Fmoc), pentane and ethyl acetate were from Fluka. All other chemicals used were of analytical grade.

Derivatization Reaction. Samples (amino acids and peptides, 10−40 nmol) were dried under reduced pressure and dissolved in 500 μL of borate buffer (0.1 M, pH 10.4). The solution was mixed vigorously with Fmoc reagent (500 μL, 5.8 mM in acetone) (Einarsson et al., 1983). The mixture was extracted two times with 2 mL of pentane/ethyl acetate (80:20). The aqueous phase containing the Fmoc derivatives was analyzed by RP-HPLC interfaced with an ESI mass spectrometer.

HPLC/Mass Spectrometry Analysis. Mass measurements and peptide fragmentation were made using a Finni-

10.1021/jf990710s CCC: $18.00
Published on Web 11/04/1999

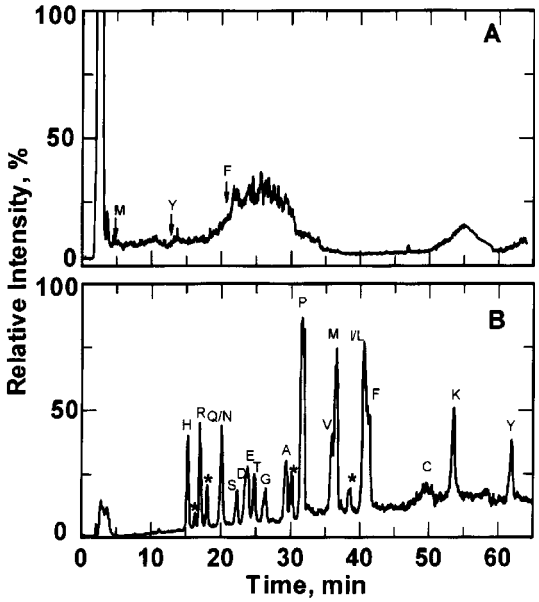

Figure 1. Total ion current profile of the HPLC/ESI-MS of amino acids: (A) underivatized amino acids (25 nmol/residue); (B) Fmoc-derivatized amino acids (500 pmol/residue). The LC/ESI-MS conditions are described under Experimental Procedures. The observed monoisotopic masses in each peak are described in Table 1. In the case of underivatized sample, amino acids were only detected following extraction of a known mass ion in the background of a high chemical noise.

ganMat ion-trap LCQ mass spectrometer interfaced with a Spectra HPLC system (FinniganMat). The system consisted of a quaternary pump (TSP P4000), an autoinjector (TSP AS3000), and a UV−vis detector (Model UVIS 205 from Linear Instruments) equipped with a high-pressure stainless steel flow cell (1.6 μL volume, 2 mm path length). The LC flow was directed to the LCQ mass spectrometer without using a flow splitter. Typically the following conditions were used: capillary temperature, 200 °C; sheath gas flow, 70 mL/min; auxiliary gas flow, 10 mL/min; source voltage, 5 kV. Other parameters were adjusted automatically during the calibration/tuning procedure as recommended by the manufacture. For MS/MS analysis of the most intense ions, the collision-induced dissociation energy was set to 35%. The mass selection and cutoff windows for the most intense ions were 1 mass unit.

Underivatized samples were analyzed using an RP C_{18} column (Nucleosil 100-3 C_{18} HD; 3 μm, 2 × 150 mm, Macherey-Nagel) with a linear gradient increase of solvent B [0.05% TFA/80% ACN (v/v) in water] in solvent A [0.045% TFA (v/v) in water] as follows: 10 min isocratic elution at 0% B, 0−25% B in 25 min, 25−50% B in 10 min, 50−100% B in 5 min followed by isocratic elution at 100% B for 5 min. The flow rate was 0.2 mL/min and detection at 215 nm. The Fmoc derivatives were separated on the same column using a linear gradient: 0−35% B in 5 min, 35−100% B in 65 min, and isocratic elution at 100% B for 5 min. The flow rate was 0.2 mL/min, and detection was accomplished at 260 nm.

RESULTS

HPLC/ESI-MS Analysis of Amino Acids. RP-HPLC in conjunction with an ESI-MS detection of a standard mixture of 17 amino acids resulted in the identification of only 3 amino acids, namely, Met, Tyr, and Phe. In addition, masses corresponding to Lys, His, and Arg were also detected in the injection peak (Figure 1). The relative abundance of the ions was low [(1−6) × 10^4], suggesting poor ionization of the analyte. HPLC/ESI-MS analysis of Fmoc-derivatized amino acids at concentrations 100−500 pmol/derivative resulted in the identification of all amino acid residues (Figure 1).

Table 1. Observed Masses of Amino Acids before and after Fmoc Derivatization from HPLC/ESI-MS Analysis

retention time, min	molecular ion, $[M + H]^+$	compound
4.9	150.0	Met
13.0	182.1	Tyr
21.1	166.0	Phe
15.0	378.1	Fmoc-His
16.7	397.2	Fmoc-Arg
19.9	355.0	Fmoc-Gln
	369.0	Fmoc-Asn
22.1	327.8	Fmoc-Ser
23.3	355.9	Fmoc-Asp
	369.8	Fmoc-Glu
23.7	341.9	Fmoc-Thr
26.1	297.7	Fmoc-Gly
29.1	311.8	Fmoc-Ala
30.1	403.8	Fmoc-Tyr
31.5	337.9	Fmoc-Pro
35.7	371.9	Fmoc-Met
36.4	339.9	Fmoc-Val
41.0	353.8	Fmoc-Leu/Ile
	387.8	Fmoc-Phe
49.4	648.9	$[Fmoc]_2$−Cys
53.4	591.0	$[Fmoc]_2$-Lys
61.7	625.8	$[Fmoc]_2$-Tyr

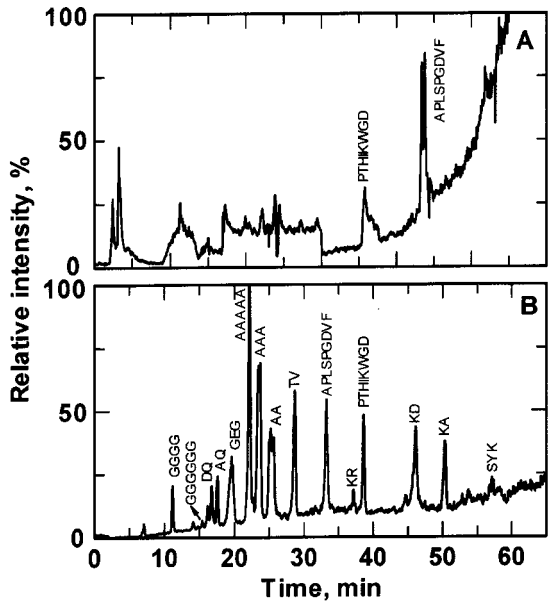

Figure 2. Total ion current profile of the HPLC/ESI-MS of synthetic peptides: (A) native peptides (1−10 nmol/peptide); (B) Fmoc-derivatized peptides (0.1−0.7 nmol/peptide). The LC/ESI-MS conditions are described under Experimental Procedures. The observed monoisotopic masses in each peak are described in Table 2.

Relative abundance of positively charged amino acid derivatives (Fmoc-Arg, Fmoc-His, Fmoc-Lys, etc.) was at least 2-fold higher compared to that of the negatively charged amino acids derivatives, for example, Fmoc-Asp and Fmoc-Glu (Figure 1). Under the conditions employed, Lys and Tyr always resulted in additional derivatization at ϵ-NH_2 and OH groups (Table 1). However, His and Arg residues carried only a single modification at the α-NH_2 group.

HPLC/ESI-MS Analysis of Peptides. The analysis of 12 synthetic peptides consisting of 2−9 residues under identical conditions used for underivatized amino acids produced similar results. The total ion current chromatogram showed only two peaks at retention times of 36 and 43.5 min and a large run through peak (Figure 2). The ESI mass spectrum corresponding to the peak

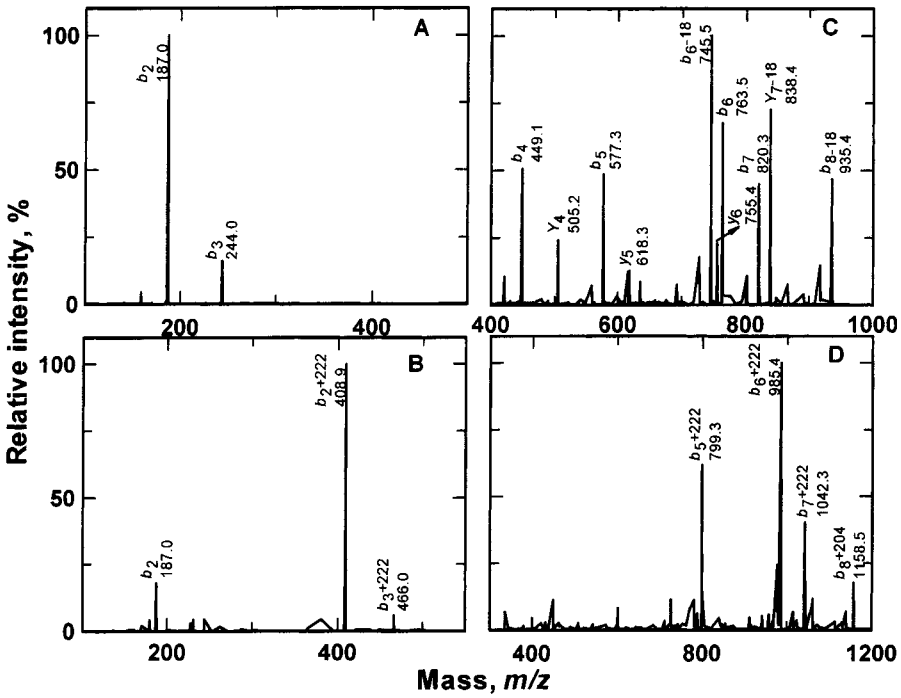

Figure 3. MS/MS fragment ion mass spectrum of the $[M + H]^+$ ion peaks of native and Fmoc-derivatized peptides: (A) product ion spectrum of $[M + H]^+ = 262.0$ peak from the native GEG; (B) product ion spectrum of $[M + H]^+ = 484.0$ peak from the Fmoc-GEG; (C) product ion spectrum of $[M + H]^+ = 953.4$ peak from the native PTHIKWGD; (D) product ion spectrum of $[M + H]^+ = 1397.3$ peak from the Fmoc-PTHIKWGD. The *b* and *y* ions retain the charges at the N and C termini, respectively.

at 36 min shows a single charged ion at *m/z* 953.4 with a distinct sodium adduct ion *m/z* 975.5 representing the peptide PTHIKWGD. The peak at 43.5 min shows *m/z* at 902.3 and its sodium adducts *m/z* 924.4 corresponding to the peptide APLSPGDVF (Figure 2). In addition, the ion current peak in the run through fraction *m/z* of three additional peptides, namely, KR (*m/z* 361.1), KD (*m/z* 262.0), and KA (*m/z* 232.0), were also detected. However, the relative abundance of ions in the run through peak was at least 2 orders of magnitude lower than that observed for the hydrophobic peptides (Figure 2). The mass detection limit of SC peptides was in the 10−100 nmol range.

The relative abundance of Fmoc-derivatized peptide ions was in the range of $(0.2-2) \times 10^7$, at least 3 orders of magnitude higher compared to that of the native peptides. The chromatographic resolution was better, and mass peaks of all the synthetic peptides were detected (Figure 2). As expected, peptides KA, KR, KD, and PTHIKWGD showed a positive mass shift of 444 due to double-derivatization at the N terminus and ϵ-NH_2 group of Lys (Table 2). In the case of SYK, in addition to the N terminus, both tyrosine and lysine residues were derivatized, showing a net positive mass shift of 666 (Table 2). The overall charge status of derivatized peptides did not change, and all of them were detected as single charged molecular ions (Table 2).

MS/MS Analysis. Fragmentation patterns of native and derivatized peptides were obtained by MS/MS analysis of the most intense ions. For illustration purposes, we selected a hydrophilic tripeptide, GEG, and a hydrophobic octapeptide, PTHIWKGD, to perform MS/MS analysis. Both show the parent molecular ion at *m/z* 262.0 and 953. 4, respectively. The product ion profile of the underivatized GEG results in fragmentation at the C-terminal Gly residue, producing a b_2 ion at *m/z* 187.0. Fmoc derivatization increased the mass of GEG by 222 (Fmoc-GEG, *m/z* 484.0). The fragmentation pattern also showed predominance of b_2 ion (Fmoc-GE, *m/z* 408.0; Figure 3). The native PTHIWKGD showed generation of both the *b*-type (b_4-b_8; *m/z* 449.1, 577.3, 763.5, 820.3, and 935.4) and *y*-type (y_4-y_7; *m/z* 505.2, 618.3, 755.4, and 838.4) ions. However, the derivatized peptide fragmentation profile showed predominantly the presence of *b*-type ions (799.3, 985.4, 1042.3, and 1158.5; see Figure 3). The b_8 ion peak (*m/z* 1158.5) was the result of a simultaneous loss of the Fmoc moiety and a water molecule from the parent ion (*m/z* 1397.3). Similar analysis of the derivatized nonapeptide, APLSPGDVF, also showed a shift to *b*-type ions in MS/MS analysis compared to the mixture of both the *b*- and *y*-type ions without the Fmoc derivatization.

Table 2. Observed Masses of Native and Fmoc-Derivatized Peptides from HPLC/ESI-MS Analysis

retention time, min	molecular ion, $[M + H]^+$	compound
35.5	953.4	PTHIKWGD
43.3	902.3	APLSPGDVF
13.8	526.0	Fmoc-QR
16.00	583.0	Fmoc-GGGGGG
16.5	484.1	Fmoc-QD
17.3	462.2	Fmoc-QA
19.3	484.0	Fmoc-GEG
21.9	596.0	Fmoc-AAAAA
23.4	454.0	Fmoc-AAA
25.2	383.0	Fmoc-AA
28.4	441.0	Fmoc-TV
33.0	1124.1	Fmoc-APLSPGDVF
36.9	747.4	$[Fmoc]_2$-KR
38.6	1397.5	$[Fmoc]_2$-PTHIKWGD
44.6	1063.1	$[Fmoc]_3$-SYK
45. 9	706.0	$[Fmoc]_2$-KD
50.1	662.1	$[Fmoc]_2$-KA

DISCUSSION

The ionization potential of peptides is highly dependent on the composition, size, and nature of the mobile

phase. The intensities of the molecular ions $[M + H]^+$ of the underivatized free amino acids and SC peptides are poor, making their identification and characterization highly tedious in LC/ESI-MS analysis (this work; Cárdenas et al., 1997). The detection levels in the negative ion mode $[M - H]^-$ mass spectrometry are also poor (Griffiths et al., 1995). Chemical modification enhances the intensities of the ions (Hunt et al., 1986; Bartlet-Jones et al., 1994; Cárdenas et al., 1997; Griffiths et al., 1995). However, most of these studies either have been carried out with off-line MS analysis or used peptides of medium to long chain lengths.

Fmoc derivatization of amino acids and peptides is highly quantitative and a well-established methodology. In conjunction with fluorescence detection, derivatized amino acids and peptides can be detected in the subpicomole range (Einarasson et al., 1983; Roturier et al., 1995). For on-line MS analysis, a major advantage of derivatization is a mass shift of the molecular ion by 222 mass units per Fmoc derivative and an overall improved ionization of peptides/amino acids even in the presence of TFA. The latter is especially helpful as it enables the retention of superior chromatographic resolution properties in the most frequently used mobile phase system in RP-HPLC. TFA is known to suppress the peptide ion abundance (Banks et al., 1994; Kay and Mallet, 1993; Eshraghi and Chowdhury, 1993). Substitution of TFA by acetic acid improves analyte ionization, but the reverse-phase separation of peptides is poor with non-reproducible retention times, poor peak shape, and no photometeric detection between 200 and 230 nm. The superior resolution by RP-HPLC enabled better identification of peptides, at least 2 orders of magnitude higher compared to that of underivatized free amino acids and peptides. Each detected peak was a singly charged species. Furthermore, additional derivatization at OH and ϵ-NH_2 groups of Tyr and Lys, respectively, not only provides direct evidence for the occurrence of these residues in peptides but also helps to differentiate between isobaric molecules, for example, Lys and Glu residues. Nevertheless, additional derivatization of Tyr and Lys can be manipulated by lowering the reaction pH to 8, at which only N-terminal Fmoc derivatives of Lys and Tyr are observed. An additional advantage of Fmoc derivatization is the preferential formation of *b*-type ions on MS/MS fragmentation. The *b*-type ions retain charge at the N terminus and are relatively less complicated in assigning a peptide sequence.

In conclusion, precolumn derivatization with Fmoc followed by RP-HPLC/ESI-MS allows easy and rapid detection and identification of short hydrophilic peptides. Food protein degradation either by fermentation or by enzymes invariably leads to the generation of a wide variety of SC peptides in relatively low concentrations. Identification of naturally occurring peptides will allow their synthesis in milligram to gram quantities by chemical means for their evaluation as taste potentiators or precursors for generation of specific flavor active compounds in model reaction systems.

ABBREVIATIONS USED

ACN, acetonitrile; ESI-MS, electrospray ionization mass spectrometry; Fmoc, 9-fluorenylmethyl chloroformate; LC/MS, liquid chromatography/mass spectrometry; RP-HPLC, reverse-phase high-performance liquid chromatography; SC, short-chain; TFA, trifluoroacetic acid.

ACKNOWLEDGMENT

We thank Drs. M. A. Juillerat and C. E. Hansen for critically reading the manuscript. We give special thanks to Dr. M. Affolter for discussions on MS analysis.

LITERATURE CITED

Anson Moye, H.; Boning, A. J. Versatile fluorogenic labeling reagent for primary and secondary amines: 9-fluorenylmethyl chloroformate. *Anal. Lett*. **1979**, *12*, 25−35.

Banks, J. F.; Quinn, J. P.; Whitehouse, C. M. LC/ESI-MS determination of proteins using conventional liquid chromatography and ultrasonically assisted electrospray. *Anal. Chem*. **1994**, *66*, 3688−3695.

Bartlet-Jones, M.; Jeffery, W. A.; Hansen, H. F.; Pappin, D. J. C. Peptide ladder sequencing by mass spectrometry using novel, volatile degradation reagent. *Rapid Commun. Mass Spectrom*. **1994**, *8*, 737−742.

Cárdenas, M. S.; Van der Heeft, E.; De Jong, A. P. J. M. On-line derivatization of peptides for improved sequence analysis by micro-column liquid chromatography coupled with electrospray ionization-tandem mass spectrometry. *Rapid Commun. Mass Spectrom*. **1997**, *11*, 1271−1278.

Einarsson, S.; Josefsson, B.; Lagerkvist, S. Determination of amino acids with 9-fluorenylmethyl chloroformate and reversed-phase high-performance liquid chromatography. *J. Chromatogr*. **1983**, *282*, 609−618.

Eshraghi, J.; Chowdhury, S. K. Factor affecting electrospray ionization of effluents containing trifluoroacetic acid for high-performance liquid chromatography/mass spectrometry. *Anal. Chem*. **1993**, *65*, 3528−3533.

Griffiths, W. J.; Lindh, I.; Bergman, T.; Sjövall, J. Negative-ion electrospray mass spectra of peptides derivatized with 4-aminonaphthahalenesulphonic acid. *Rapid Commun. Mass Spectrom*. **1995**, *9*, 667−676.

Izzo, H. V.; Ho, C. T. Peptide-specific Maillard reaction products: A new pathway for flavor chemistry. *Trends Food Sci. Technol*. **1992**, *3*, 253−257.

Kay, I.; Mallet, A. Use of an on-line liquid chromatography trapping column for the purification of proteins samples prior to electrospray mass spectrometric analysis. *Rapid Commun. Mass Spectrom*. **1993**, *7*, 744−746.

Roturier, J. M.; Le Bars, D. Separation and identification of hydrophilic peptides in dairy products using FMOC derivatization. *J. Chromatogr. A* **1995**, *696*, 209−217.

Received for review June 28, 1999. Revised manuscript received September 16, 1999. Accepted September 20, 1999.

JF990710S