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DIRECT LIQUID INTRODUCTION MASS SPECTROMETRY OF SOME UNDERIVATIZED DIPEPTIDES AND POLYPEPTIDES*

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SUMMARY

A series of underivatized dipeptides containing proline as either the N- or C-terminal amino acid, and a series of underivatized peptides containing two to six glycines have been studied. The complexity and position of the second amino acid, the mode of ionization and the detectability of characteristic fragments were examined.

All the dipeptide spectra exhibited two fragment ions that are characteristic of their molecular weight, but characteristic fragments that would permit the determination of their structure were not always observed. Molecular weight information in the $(Gly)_n$ spectra was only present up to the tripeptide. When the number of amino acids was larger, only a small and uncharacteristic fragment ion (highly stabilized) was observed.

It is concluded that, under presently available conditions, the unequivocal determination of the primary structure of a mixture of underivatized small peptides is not possible by direct liquid introduction liquid chromatography–mass spectrometry.

INTRODUCTION

The elucidation of the primary structure of unknown peptides in a mixture is of great importance, especially in the study of food and nutrition. Mass spectrometry (MS) is the method of choice, but it has to be coupled with a separation technique such as gas chromatography (GC), liquid chromatography (LC) or MS itself, either on- or off-line. The classical procedure involves enzymatic degradation of the peptides, followed by separation and identification of the sequence amino acids by GC–MS after derivatization and alignment of the amino acids[1]. Recently developed MS techniques permit the shortening of this procedure by obtaining the sequence directly either by fast atom bombardment (FAB) mass spectrometry[2] or by the coupling of LC and MS[3,4]. Even more recently, enzymatic degradation has been tentatively used on-line with the separation and identification steps by thermospray LC–MS[5].

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The aim of this study was to determine to what extent direct liquid introduction (DLI) LC–MS coupling could be used to ascertain the primary structure of dipeptides or small peptides in an unknown mixture produced, for example, after digestion of polypeptides by dipeptidyl peptidases. As a first step, we studied a series of dipeptides and small polypeptides. The dipeptides all contained the amino acid proline (Pro), the second amino acid was either N-terminal or C-terminal with increasing complexity. Different aspects were examined: the position of the second amino acid relative to Pro, the nature of the second amino acid, the mode of ionization (positive or negative), and the effect of an added reactant gas. The polypeptides contained only glycine, $(Gly)_n$ with $n = 2$–6.

EXPERIMENTAL

Chemicals

All the peptides were purchased from Bachem (Switzerland). Methanol (Merck, Switzerland) was of analytical-reagent grade and used without further purification. Water was doubly distilled.

Liquid chromatography

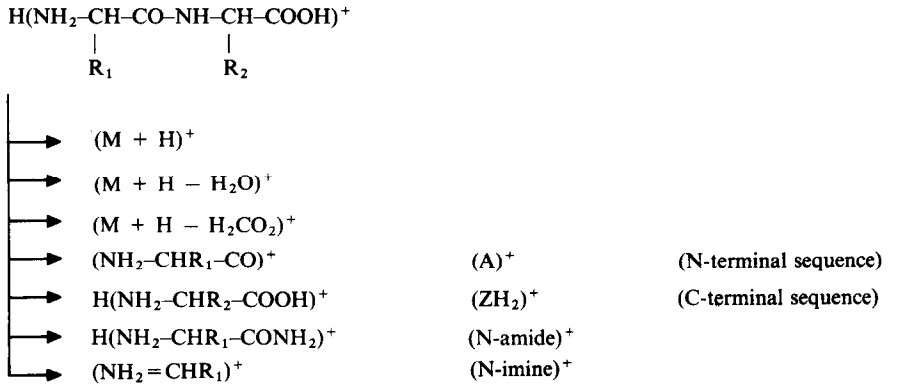
A Waters (U.S.A.) 6000A pump equipped with an Altex 210 20-μl loop injector was used. The system was employed as a liquid injector to the mass spectrometer without a column. The mobile phase was a mixture of water and methanol (1:4) at a flow-rate of 0.5 ml/min.

Mass spectrometry

A Nermag (France) R10-10C quadrupole system was coupled to the LC via a DLI interface equipped with the Nermag desolvation chamber[6]. Spectra were obtained in either positive or negative chemical ionization (CI) mode. To reach optimum conditions, the flow-rate into the ion source was kept at 10 μl/min (split ratio,

TABLE I

CHARACTERISTIC FRAGMENTATION PATTERN OF A DIPEPTIDE IN THE POSITIVE CI MODE



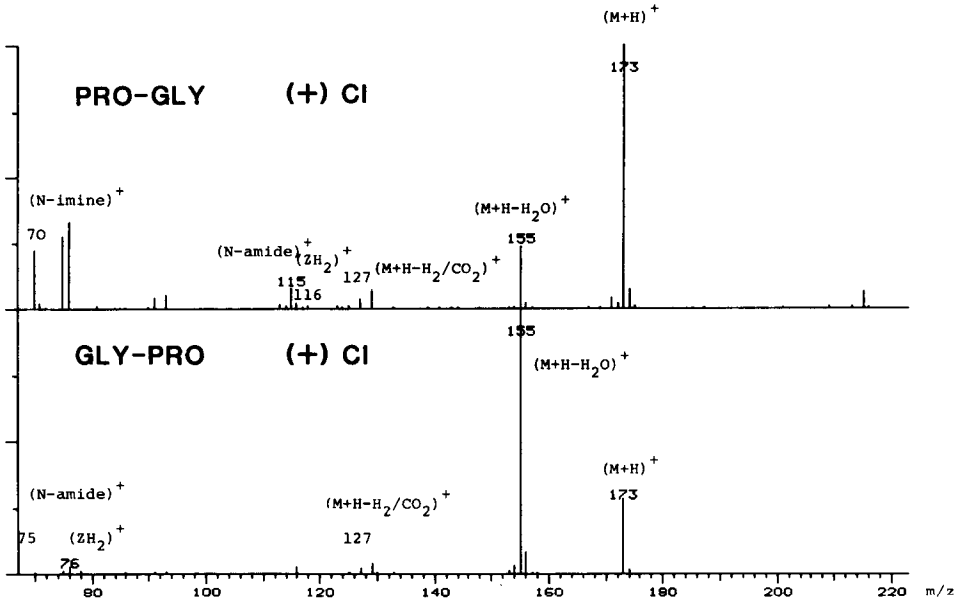


Fig. 1. Mass spectra of Pro-Gly and Gly-Pro obtained by direct liquid introduction in positive CI mode.

1:50). The temperatures were maintained at 200°C in the desolvation chamber and 150°C in the ion source.

The amount of substance introduced into the ion source was *ca.* 1 μg, dissolved in the mobile phase.

RESULTS

Dipeptides

The CI-MS fragmentation pattern of a dipeptide can be schematically repre-

TABLE II

RELATIVE INTENSITIES (REFERENCE: BASE PEAK) OF THE CHARACTERISTIC FRAGMENT IONS OF ALL THE DIPEPTIDES STUDIED IN THE POSITIVE CI MODE

	$(M + H)^+$ (%)	$(M + H - H_2O)^+$ (%)	$(M + H - H_2CO_2)^+$ (%)	$(A)^+$ (%)	$(ZH_2)^+$ (%)	$(N\text{-}amide)^+$ (%)	$(N\text{-}imine)^+$ (%)
Pro-Gly	100	25.1	3.7	–	35.4	9.1	22.5
Pro-Ala	100	44.5	11.9	–	–	17.4	19.1
Pro-Val	100	17.6	22.6	2.8	1.2	17.5	78.4
Gly-Pro	50.4	100	2.2	1.0	3.3	1.2	n.d.*
Ala-Pro	36.3	100	4.5	0.6	6.8	–	n.d.
Leu-Pro	100	50.0	2.3	2.5	5.6	1.7	14.9
Met-Pro	0.2	100	–	–	0.2	–	0.2

* n.d. = Not determined.

sented as shown in Table I. If the structure of an unknown dipeptide is to be determined, the molecular weight [given by the $(M + H)^+$ and/or the $(M + H - H_2O)^+$ ions] and some of these characteristic fragment ions should be observable.

Fig. 1 shows, as an example, the spectra of Pro-Gly and Gly-Pro, and Table II gives the intensities relative to the base peak of these characteristic fragment ions for all the peptides studied.

Fig. 2 shows, as an example, the spectra of Pro-Ala and Ala-Pro in both positive and negative CI modes, and Table III gives the relative intensities of both the pseudo-molecular ions and those corresponding to the loss of one molecule of water.

Polypeptides

The characteristic fragment ions we considered were only $(M + H)^+$ and $(M + H - H_2O)^+$ ions and those arising from the cleavage of the amide linkages, the so-called $(A_n)^+$ and $(Z_nH_2)^+$ ions.

Their relative intensities are listed in Table IV for the five polypeptides studied, $(Gly)_2$ to $(Gly)_6$.

DISCUSSION

Twenty different amino acids containing very different chemical functions are found in common proteins and polypeptides. They can form twenty different homo-

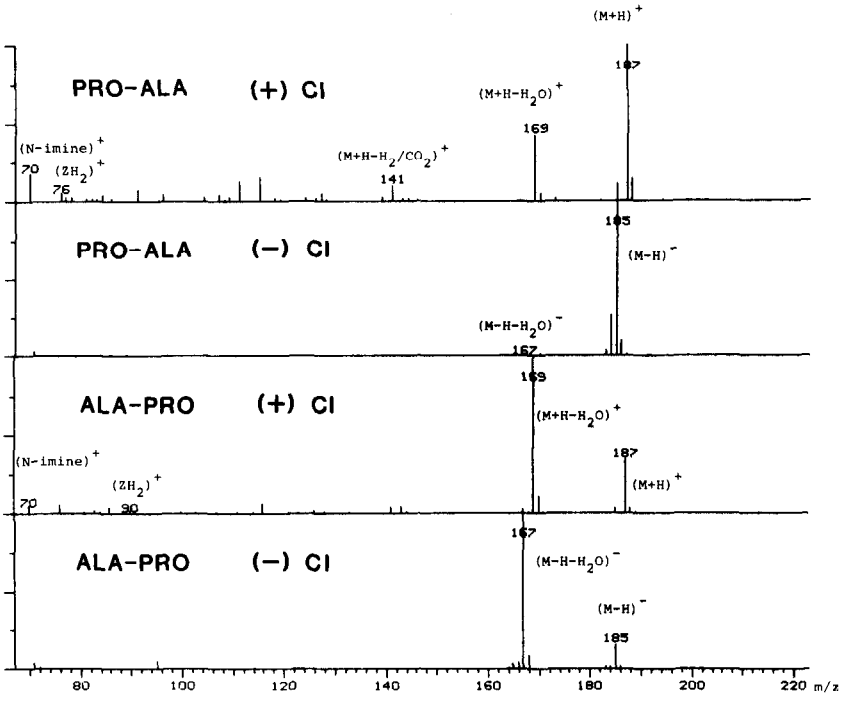


Fig. 2. Mass spectra of Pro-Ala and Ala-Pro obtained by direct liquid introduction in positive and negative CI modes.

TABLE III

RELATIVE INTENSITIES (REFERENCE: BASE PEAK) OF THE (PSEUDO-MOLECULAR)$^{+/-}$ AND (PSEUDO-MOLECULAR $- H_2O)^{+/-}$ IONS OF ALL THE DIPEPTIDES STUDIED IN THE POSITIVE AND NEGATIVE CI MODES

		(Pseudo-molecular)$^{+/-}$ (%)	*(Pseudo-molecular $- H_2O)^{+/-}$ (%)*
Pro-Gly	(+)CI	100	125.1
	(−)CI	100	9.2
Gly-Pro	(+)CI	50.4	100
	(−)CI	100	27.1
Pro-Ala	(+)CI	100	44.5
	(−)CI	100	41.8
Ala-pro	(+)CI	36.3	100
	(−)CI	17.3	100
Pro-Val	(+)CI	100	17.6
	(−)CI	100	35.1
Leu-Pro	(+)CI	100	7.3
	(−)CI	100	16.6
Met-Pro	(+)CI	0.2	100
	(−)CI	–	100

TABLE IV

RELATIVE INTENSITIES (REFERENCE: BASE PEAK) OF THE CHARACTERISTIC FRAGMENT IONS OF THE POLYPEPTIDES STUDIED IN THE POSITIVE CI MODE

	$(M + H)^+$		*$(M + H - H_2O)^+$*		*$(A_n)^+$*		*$(Z_nH_2)^+$*	
	m/z	*%*	*m/z*	*%*	*m/z*	*%*	*m/z*	*%*
$(Gly)_2$	133	100	115	32.7	58	n.d.*	76	14.7
$(Gly)_3$	190	100	172	2.0	58	n.d.	133	35.4
					115	17.7	76	32.8
$(Gly)_4$	247	25.5	229	4.7	58	n.d.	190	14.4
					115	100	133	76.9
					172	18.2	76	–
$(Gly)_5$	304	–	286	–	58	n.d.	247	–
					115	100	190	–
					172	–	133	–
					229	–	76	4.9
$(Gly)_6$	361	–	343	–	58	n.d.	304	–
					115	100	247	–
					172	0.2	190	–
					229	–	133	–
					286	–	76	5.7

* n.d. = Not determined.

geneous dipeptides and 190 different heterogeneous dipeptides. Moreover, in proteins and polypeptides, uncommon derivatives can also be found. This situation makes the determination of the primary structure of dipeptides very difficult and enhances the potential advantages of MS. As is the case for other classes of compounds (steroids, for example) it would be of great interest to find general rules governing the fragmentation of these dipeptides and thus enable the use of computer programs for the resolution of their primary structure[7].

Before discussing these results, we would like to briefly mention the problems we encountered and the limitations provoked both by the technique itself and by the conditions we chose voluntarily.

Problems encountered

We used the DLI interface as a liquid injector without column separation mainly because in our system a nickel diaphragm is employed, which does not permit the use of acidic mobile phases. We hope soon to be able to circumvent this limitation. One drawback in this method is that some free amino acids might be present as impurities in the dipeptides and thereby produce ions that may be misinterpreted as originating from the dipeptides.

Another drawback of the DLI interface is the high background produced by the solvent mixture, raising the minimum m/z value observable to 70.

But the main problem appears to be the reproducibility of the spectra. In DLI LC–MS, the recorded spectra are very sensitive to pressure and temperature in the ion source. We therefore tried to keep these parameters as constant as possible in order to obtain comparable spectra. For this reason, during the study the conditions were not changed for the different dipeptides. They were therefore not always optimal and so the most characteristic spectrum was not necessarily obtained in each case. In fact, in a real case with an unknown mixture, such an optimization would be difficult during a chromatographic run and we wanted keep as close to reality as possible.

Dipeptides

Position and nature of the second amino acid. The base peak is always either the $(M + H)^+$ or the $(M + H - H_2O)^+$ ion. Owing to the specific structure of Pro, when Pro is N-terminal, the base peak is always the $(M + H)^+$ ion. In all cases except the Met-containing dipeptide, the other ion is rather abundant, thus permitting the dipeptide molecular weight to be determined.

The abundances of the other characteristic fragment ions are variable and do not appear to be reliable enough for the structure to be ascertained.

When Pro is N-terminal, the $(N\text{-imine})^+$ ion is probably highly stabilized and rather abundant. The $(N\text{-imine})^+$ ion m/z values were too small to be detected, owing to the high background observed in DLI LC–MS, when a small amino acid is N-terminal (Gly or Ala).

The spectra of the sulphur-containing dipeptide, Met-Pro, exhibit only one main ion, $(M + H - H_2O)^+$.

From these observations, it appears that the fragmentation pattern does not vary regularly in a series of dipeptides.

Mode of ionization. As shown in Fig. 2, positive and negative CI produce the

same main fragment ions [except for Gly-Pro: in negative CI the base peak is the $(M - H)^-$ ion]. However, in negative CI, there is less background and the spectra look "clearer". Apart from the fragment ions corresponding to the molecular weight, there are hardly any characteristic fragments. Moreover, the absolute intensities of the ions are higher, thus providing more sensitivity. This suggests that negative CI would be more useful in quantitative measurement than in structure determinations.

Effect of an added reactant gas. We tried to add several of the usual reactant gases used in CI-MS: methane, $i\text{-}C_4H_{10}$ and ammonia with the aim of increasing and stabilizing the fragmentation pattern. But because of the pressure in the ion source necessary to optimize the DLI interfacing, the pressure of the added reactant gas has to remain low, and no change could be observed in the fragmentation pattern.

Polypeptides

When studying the polypeptides $(Gly)_n$, we only took into account the $(M + H)^+$, $(M + H - H_2O)^+$, $(A_n)^+$ and $(Z_nH_2)^+$ ions because the (N-imine)$^+$ and (N-amide)$^+$ ions were not abundant enough to be observed under our conditions. For $n = 2$ and 3, the base peak was the pseudo-molecular ion and all the characteristic fragment ions could be observed. But, for $(Gly)_4$, although an abundant peak was observed for $(M + H)^+$, the base peak corresponded to the $(A_2)^+$ ion, characteristic of the $(Gly)_2$ moiety. However, other characteristic fragments that enabled the identification of the peptide were present.

For $n = 5$ and 6, the only observable peak was at m/z 115, corresponding to the $(A_2)^+$ ion. It is suggested that this ion is so highly stabilized that it is the only observable ion under these conditions.

CONCLUSIONS

These observations suggest that it is difficult to use DLI-LC–MS to determine the primary structure of a mixture of unknown dipeptides in a single run. Although in all the dipeptides studied some information was available on the molecular weight, the fragmentation pattern is too unpredictable. Certainly, we and other authors have obtained LC–MS spectra of larger known peptides showing characteristic fragment ions that permit their identification. In this study, our point of view was to imagine ourselves in the situation of analysing an unknown mixture and of having to use average conditions, keeping them constant during the whole experiment. An elegant solution would be to combine this interfacing technique with MS–MS where CID would provide the necessary information. Some other problems currently encountered in this stype of determination, the distinction between Leu and Ile for example, would also be solved, as shown by Bradley *et al.*[8]. However, LC–MS–MS appears to be valid enough for small peptides only, probably up to four to six amino acid residues. For larger peptides, the solution could be provided by FAB-MS–MS, although it is not (yet?) an on-line technique.

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