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Electrospray Mass Spectrometric Study of Melittin Trypsinolysis by a Kinetic Approach

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The kinetics of tryptic digestion of melittin was studied by combined electrospray ionization time-of-flight mass spectrometry and high-performance liquid chromatography. The ratios of the kinetic constants for cleavage of the peptide bonds that are susceptible to trypsin action were determined. It is shown that trypsin does not manifest affinity for the hydrolysis of the peptide bonds inside the Arg,Lys cluster series as efficiently as it cleaves the peptide at the separately localized Lys residue. This feature demonstrates clearly the advantage of the kinetic approach to tryptic mapping of proteins. The kinetic approach allows the determination of not only discrete structural segments in protein structure but also their relative locations and their amino acid sequences. Using the melittin digests and some artificially prepared amino acids and dipeptides mixtures as models, it is shown that the presence and nature of basic amino acids predetermines the charge states of the molecules analyzed by electrospray but not the yields of their ions. The aliphatic parts of the molecules seem to be more important in determining the actual ion yields.

Tryptic digestion is often used for protein and peptide mapping.[1-3] Protein (or peptide) tryptic digestion results exclusively in highly specific cleavage of peptide bonds near the carboxylic group of lysine (Lys) and arginine (Arg) residues. As a rule, its complete digestion is used for protein mapping. The presence of peptides that are products of incomplete protein cleavage hampers the application of such analytical techniques as HPLC or electrophoresis. At the same time, these products can be easily determined when the digest is analyzed by electrospray ionization mass spectrometry (ESI-MS).[4]

Proteins often contain not only separate residues of Arg and Lys but also segments consisting of repetitive basic residues. These repetitive segments are common features of the primary structure of histones,[5] ribosomal proteins,[6] and some other proteins.[7,8]

The mapping of such proteins using complete tryptic digestion results in formation of very short peptide fragments[1] and loss of structural information due to destruction of the cluster sequences. It is impossible to find the overlapping sequences using these short fragments, and thus protein primary structure determination cannot be successfully accomplished. For mapping of such proteins, it will be better to use incomplete tryptic digestion in combination with ESI-MS. Such ESI-MS mapping allows not only the recognition of small structural segments in protein structures but also the determination of their relative locations and their amino acid sequences. It is difficult to choose suitable digestion conditions for these proteins since the ratios of the hydrolysis rates for peptide bonds formed by isolated and cluster Arg and Lys residues still remain unexplored.

We chose melittin (MEL) as a model to perform the kinetic study of trypsinolysis processes of a substrate containing both separate and cluster Arg,Lys residues. We have used ESI-MS to identify proteolysis products both in unfractionated digests and in the individual chromatographic fractions. The concentrations of individual trypsinolysis products were determined by HPLC.[9]

Another goal of our work was to estimate the possibility of the direct quantitative ESI-MS analysis of the peptide digests. The application of ESI-MS to the kinetic quantitative measurements was first shown in ref 10. Tryptic digestion of the ethyl ester of benzoylarginine was studied in the on-line mode. The ESI-MS technique has several advantages when applied to the kinetic measurements: (i) the mass spectrum pattern is not influenced by the acquisition time and (ii) the term of concentration could be used because the analyzed liquid sample is injected into the instrument.[11-13] However, the intensities of peaks in mass spectrum are not strictly proportional to the amount of compo-

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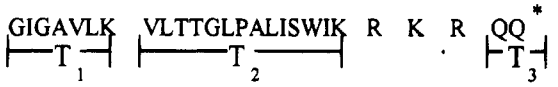
Chart 1. Structural Blocks in MEL Primary Structure



nents in the sample. The relative abundances of the detected ions are dependent on their m/z values, and the spectrum pattern might be strongly influenced by the actual acquisition regimes, namely the value of nozzle/skimmer voltage and the voltage applied to a spraying capillary. Moreover, the relative abundances of peptide ions are known to be strongly influenced by the peptide amino acid compositions. That is why the series of ESI-MS spectra of the reaction mixtures could be used only for qualitative interpretation. These data allow one to monitor the dynamics of the decreasing or increasing of the concentration of specified analyte.[14,15] However, the peak intensities could be used for quantitative estimations in special cases.

In this paper, the approaches to quantitative analysis of peptide mixtures are developed using melittin tryptic digests as the test mixtures. The pool of peptides having the same N-terminus part and differing by the absence of some amino acid residues at the C-terminus part could be yielded by melittin trypsinolysis. This allows one to compare the relative abundances of their ions with the concentrations independently determined by HPLC. The obtained results were also compared with those of the ESI-MS analysis of model mixtures containing amino acids and dipeptides.

MATERIALS AND METHODS

Trypsin from bovine pancreas was purchased from Spofa (Czechoslovakia). The actual concentration of active trypsin was determined as described in ref 16.

Melittin was purchased from Sigma (St. Louis, MO).

MEL was digested by trypsin at 25 °C in 0.1 M Tris buffer adjusted to pH 8.0 by HCl. The initial concentration of MEL was 0.8 mM, and the enzyme/substrate ratio was 1:500 (w/w), accounting for the active enzyme. At selected times, the digestion was stopped by the addition of 10% acetic acid.

MEL digests were separated on a Milikhrom liquid chromatograph (Nauchpribor, Russia Federation) using 2 × 62 mm stainless steel columns, packed with Nucleosil 5 C_{18} (Macherey Nagel). The elution was carried out with a 20–50% (v/v) gradient of component B in A + B mixture. Component A was 0.05% TFA in water; component B was 0.05% TFA in acetonitrile.

The concentrations of proteolysis products were estimated from peak area data using the coefficients of extinction calculated in accordance with the additive scheme.[17]

Mass spectra were recorded on an experimental prototype of a time-of-flight reflectron mass spectrometer equipped with an electrospray ion source. A detailed description of instrument's construction and its operational principles was given in ref 4.

Singly and doubly protonated ions of gramicidin S having m/z values 1142.5 and 571.5, respectively, were used for the instrument calibration. All peak masses were isotopically averaged. Routine mass accuracy was better than 0.02%.

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Table 1. Mass Spectrometric Analysis of the MEL Tryptic Digest

digestion product	ions detected, m/z[a]	molecular weight: measured	molecular weight: expected
MEL	949.8(3); 712.6(4)	2846.4	2846.5
T_1T_2-RKR	648.8(4)	2591.2	2591.2
T_1T_2-RK	487.9(5)	2434.5	2435.0
T_1T_2-R	769.9(3)	3206.7	3206.9
T_1T_2	1076.3(2)	2150.6	2150.7
T_2-RKR-T_3	1104.8(2)	2207.6	2207.7
T_2-R	835.0(2); 557.1(3)*	1668.2	1668.1
T_2	657.8(1); 329.5(2)*	656.9	656.8
KR-T	279.8(2)*	557.6	557.6
R-T_3	430.4(1)	429.4	429.5
KR	303.2(1)*	302.2	302.4
RKR	459.7(1); 230.4(2)*	458.7	458.6
T_3	274.1(1)*	273.1	273.3

[a] The ions were detected using nozzle/skimmer voltage (U_{ns}) of 200 V. The ions designated by asterisks were detected using U_{ns} = 150 V.

An integrating transient recording system was used for spectra acquisition: 30 000 transients were accumulated for each mass spectrum during a 0.5 min acquisition period. Routine peptide concentrations were about 0.01–0.1 mM, so 10–100 pmol of the each samples was required for the analysis.

For spectra acquisition, individual fractions obtained from HPLC were vacuum dried and redissolved in 15 mL of 2% acetic acid/acetonitrile (1:1 v/v) and injected at a flow rate of 2 mL/min. Nozzle/skimmer voltage was 200 V if not otherwise specified.

The samples for ESI-MS analysis of unfractionated digests were prepared using the same enzyme/substrate ratio and hydrolysis conditions. The initial concentration of MEL was 0.18 mM in these cases. The samples acidified by 10% acetic acid were diluted with acetonitrile in a 1:5 ratio prior to the MS analysis. As it was shown in the special HPLC experiment, these changes in the digestion conditions did not influence the relative concentrations of products compared with those previously observed ones.

The digests for quantitative measurements were desalted using C_{18} chromatographic columns prior to the MS analysis. The adsorbed peptides were washed with 10% acetonitrile to remove buffer components and low molecular weight impurities, followed by elution with 60% acetonitrile. The yield of peptide products was tested by HPLC data and appeared to be quantitative for all the digestion products, with the exceptions of the extremely hydrophilic T_1 fragment and short peptides that were completely lost.

RESULTS AND DISCUSSION

MEL contains one separate Lys residue in the seventh position and a cluster of basic residues (Lys-Arg-Lys-Arg) in positions 21–24 of its sequence (Chart 1). Three structural blocks (T_1, T_2, T_3) corresponding to complete tryptic digestion can be predicted on its primary structure. We shall designate as T_x-Y (Y-T_x) the T_x fragment of the MEL sequence, where Y indicates Arg or Lys residue attached to its C- (N-) terminus. * represents the Gln-amide residue.

MEL was subjected to trypsin cleavage, and the resulting digest was analyzed by ESI-MS. The products formed by the hydrolysis of peptide bonds at the C-termini of all Arg and Lys residues were detected (Table 1).

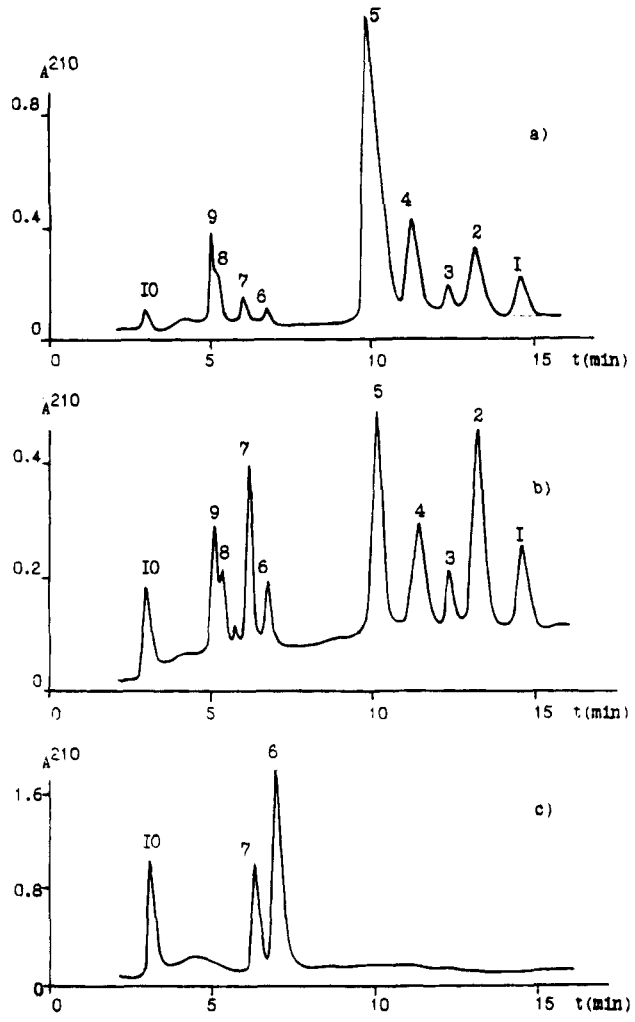
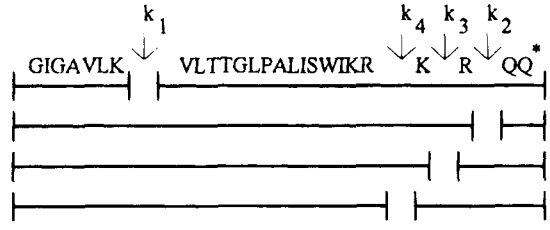


Figure 1. Chromatogram of the reaction mixture obtained by trypsin digestion. Time of hydrolysis: (a) 10 min, (b) 23 min, and (c) 10 h.

Scheme 1. Formation Pathways of Primary Products of MEL Tryptic Digestion



From the set of products obtained, one can pick out four pairs that can be related to the products of primary MEL cleavage (Scheme 1). The results of this work show that trypsin cleaves peptide bonds near separate and clustered Arg,Lys residues with comparable rates. All the other products detected (including T_2-R) are formed by further cleavage of primary products.

To determine the trypsinolysis rates of MEL and some of its digestion products, the hydrolysates were subjected to reversed phase HPLC separation (Figure 1). Chromatographic peaks were identified by MS. The results are shown in Table 2.

It should be mentioned that the results of direct ESI-MS analysis of the digests are in fairly good agreement with HPLC data. Only C-terminus digestion products, which might be eluted near the dead volume of the reversed phase column, remain unidentified by HPLC analysis.

Table 2. MS Identification of the Chromatographic Peaks of hydrolysates of Melittin

peak number[a]	molecular weight measured	molecular weight expected	identification
1	2151.2	2150.7	T_1T_2
2	2307.2	2306.9	T_1T_2-R
3	2435.4	2435.0	T_1T_2-RK
4	2591.9	2591.2	T_1T_2-RKR
5	2846.5	2846.5	MEL
6	1511.7	1511.9	T_2
7	1668.7	1668.1	T_2-R
8, 9	1952.6	1952.4	T_2-RKR
	2208.1	2207.7	T_2-RKR-T_3
10	657.0	656.8	T_1

[a] Peaks are numbered in accordance with Figure 1 and designated in accordance with Chart 1.

The concentrations of digestion products were calculated from peak area data, taking into account their extinction coefficients. It was shown previously that the extinction coefficients of individual aromatic amino acids (Tyr, Phe, His, and Trp) as well as that of the peptide bond, could be used as increments for additive scheme calculation of the molar extinction coefficient value for a 1–3 kDa peptide.[17,18] Consequently, HPLC with spectrophotometric detection of an eluate seems to be a good technique for performing these measurements. Concentrations of all digestion products could be calculated from their peak area data without employing any additional techniques (for instance, amino acid analysis). Application of the ratios of extinction coefficients for chromatographic peak normalization allows one to avoid the influence of systematic error connected with the geometry of the detector cell as well as the error in a loading sample volume. The error for the concentration determination would not exceed 10% of the measured concentration value. We assume that this is quite sufficient accuracy considering the specific goals of this study.

The ratio of cleavage rates of separate and "cluster" peptide bonds could be evaluated from (1) the total rate of MEL digestion and (2) the rate of T_1 peptide formation, assuming that cleavage rate constants are not affected by the cleavage of other bonds (e.g., T_1T_2, T_1T_2-R, T_1T_2-RK, and T_1T_2-RKR are assumed to have the same cleavage rate constant, k_1, in forming T_1).

According to the primary cleavage of MEL (Scheme 1), the rate of the disappearance of MEL and the rate of the formation of T_1 peptide may be written as follows:

$$\frac{dM}{dt} = -(k_1 + k_2 + k_3 + k_4)M \quad (1)$$

$$\frac{dT_1}{dt} = k_1(M + T_1T_2\text{-RKR} + T_1T_2\text{-RK} + T_1T_2\text{-R} + T_1T_2) \quad (2)$$

where M is the MEL concentration and T_1, T_1T_2, T_1T_2-R, T_1T_2-RK, and T_1T_2-RKR are the concentrations of corresponding

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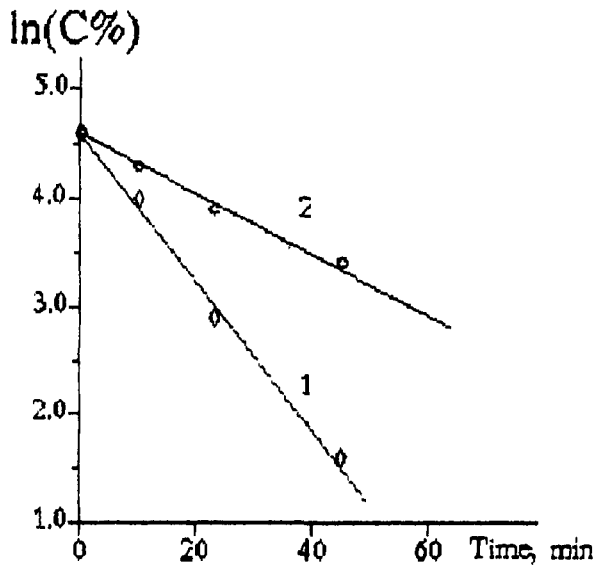

Figure 2. Total rate of melittin digestion (1) and rate of T_1 peptide formation (2) expressed in semilogarithmic coordinates.

trypsinolysis products. The initial concentrations of MEL and T_1 peptide are $[M]_{t=0} = M_0$ and $[T_1]_{t=0} = 0$, respectively. The kinetic constants k_i are designated in accordance with Scheme 1. We assume trypsin concentration is constant during the reaction and the concentrations of all substrate exceed K_m values.

It follows from eq 1 that the concentration of MEL is

$$M = M_0 e^{-(k_1+k_2+k_3+k_4)t} \quad (3)$$

Using the equation of balance,

$$M_0 = M + T_1T_2\text{-RKR} + T_1T_2\text{-RK} + T_1T_2\text{-R} + T_1T_2 + T_1 \quad (4)$$

and eq 2, one can write

$$T_1 = M_0(1 - e^{-k_1t}) \quad (5)$$

The value of the kinetic constant calculated using the slopes of the curves of MEL (1) and $M_0 - T_1$ (2), presented in semilogarithmic coordinates in Figure 2, shows that the cleavage rates between separate and cluster bonds are really similar:

$$\frac{k_2 + k_3 + k_4}{k_1} = 1.5$$

This means that the high affinity of trypsin for peptide bonds inside the Lys,Arg cluster sequences, previously found for some proteins,[7,19,20] is not observed for MEL.

To analyze the digestion pathways of MEL primary cleavage products, the ratios of hydrolysis rates of the "cluster" bonds after the splitting out of the C-terminus fragment (T_3) were estimated.

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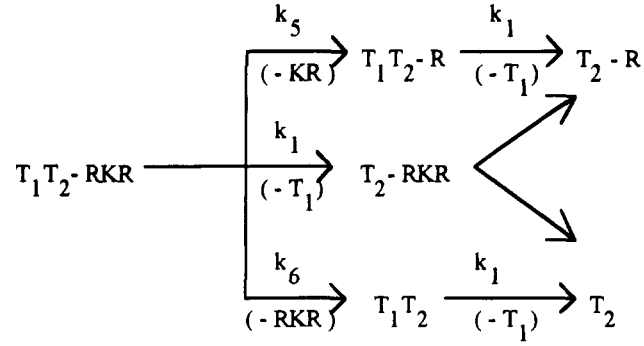
Scheme 2. Trypsinolysis Pathways of the T_1T_2-RKR Peptide



To study these processes, two peptides, T_1T_2-RKR and T_1T_2-R, were purified by HPLC from a total digest. Their subsequent trypsin treatment was carried out under the same conditions as for native MEL. In accordance with the chromatographic data (not shown), Scheme 2 for T_1T_2-RKR typsinolysis pathways could be proposed.

According to Scheme 2, showing the tripsynolisis of the T_1T_2-RKR peptide, one can write the following equations:

$$\frac{dT_1T_2\text{-RKR}}{dt} = -(k_1 + k_5 + k_6)T_1T_2\text{-RKR} \quad (6)$$

$$\frac{dT_1T_2\text{-R}}{dt} = k_5T_1T_2\text{-RKR} - k_1T_1T_2\text{-R} \quad (7)$$

$$\frac{dT_1T_2}{dt} = k_6T_1T_2\text{-RKR} - k_1T_1T_2 \quad (8)$$

$$\frac{dT_1}{dt} = k_1(T_1T_2\text{-RKR} + T_1T_2\text{-R} + T_1T_2) \quad (9)$$

where T_1, T_1T_2 , T_1T_2-R, and T_1T_2-RKR are the concentrations of the corresponding trypsinolysis products. The initial conditions of this system are

$$[T_1T_2\text{-RKR}]_{t=0} = T_1T_2\text{-RKR}_0, \quad [T_1T_2\text{-R}]_{t=0} = 0$$
$$[T_1T_2]_{t=0} = 0, \quad [T_1]_{t=0} = 0 \quad (10)$$

It follows from eqs 6 and 9 (by analogy with MEL) that

$$T_1T_2\text{-RKR} = T_1T_2\text{-RKR}_0 e^{-(k_1+k_5+k_6)t} \quad (11)$$

$$T_1 = T_1T_2\text{-RKR}_0(1 - e^{-k_1t}) \quad (12)$$

Using the slopes of the curves of T_1T_2-RKR (1) and T_1T_2-RKR_0 – T_1 (2), presented in semilogarithmic coordinates in Figure 3, the following ratio of the kinetic constant was obtained:

$$\frac{k_5 + k_6}{k_1} = 1.3$$

As it follows from eqs 7 and 8,

$$\frac{T_1T_2\text{-R}}{T_1T_2} = \frac{k_5}{k_6} = 1.4$$

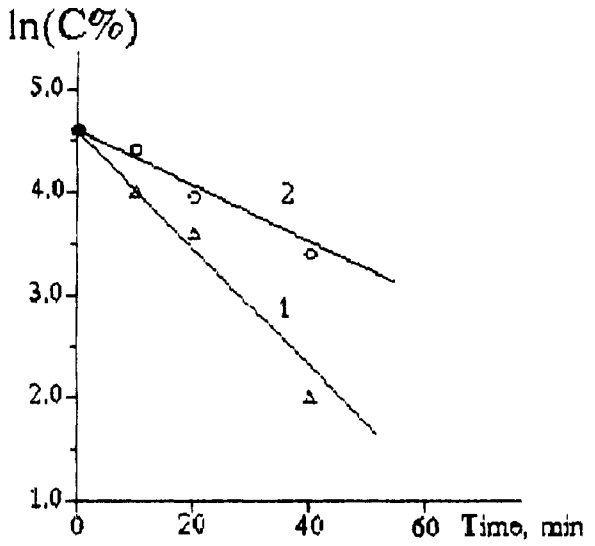


Figure 3. Total rate of T_1T_2-RKR peptide digestion (1) and rate of T_1 peptide formation (2) expressed in semilogarithmic coordinates.

At the final stage of MEL tripsynolysis, there is only one intermediate product (T_2-R) of hydrolysis which can convert to the T_2 peptide; this is the slowest stage in all of the processes studied. The half-time of the T_2-R peptide calculated using the change of its concentration by HPLC data [51% at 3 h (Table 3) and 28% at 10 h (Figure 1c)] is ~8 h.

All the data obtained—the ratios of concentrations of T_1T_2 series products (T_1T_2, T_1T_2-R, T_1T_2-RK, and T_1T_2-RKR) produced by primary and secondary cleavages—those that the k_2 value is the largest among the kinetic constants related to cluster bonds hydrolysis. At the same time, the rates of the hydrolysis pathways yielding di- and tripeptides (RKR, RK, KR) are more than an order of magnitude higher those yielding free amino acids (R, K). It should be mentioned that the absence of the RKR-T_3 peptide in the mass spectrum of the unfractionated digest points out that the observed product T_1T_2 has been yielded by secondary cleavage. The kinetic data for T_1T_2-RKR digestion indicate C-terminal dipeptide splitting from T_1T_2-RKR as the main pathway yielding T_1T_2-R. The ratios of the kinetic constants evidently show that the formation of overlapping tryptic fragments is possible even when clustered and separate Arg,Lys are simultaneously present in the protein sequence. Using MEL as a model, we have shown that monitoring MEL tryptic digestion by ESI-MS enable not only the determination of a specific structural fragment in its sequence but also the determination of its location. Application of only one enzyme (trypsin) proved to be quite sufficient in this case to obtain a representative peptide map:

It seems to us also attractive to examine whether concentrations of individual proteolysis products can be adequately estimated by ESI-MS analysis of the tryptic digests. ESI-MS data obtained for MEL digests with different degrees of conversion were verified by HPLC (Table 3). In these experiments, we used the digests desalted on a C_{18} chromatographic column prior to their MS analysis (see Materials and Methods). Our attempts to acquire MS spectra directly from the digests samples containing 0.1 M Tris buffer failed since the analyte spraying appeared to be highly fluctuating in these cases. The actual composition of the digests after their desalting was monitored by HPLC.

The ion yields for the MEL and the T_1T_2 series peptides (T_1T_2, T_1T_2-R, T_1T_2-RK, and T_1T_2-RKR) appeared to be maximal in comparison with the T_2 ones (T_2, T_2-R, T_2-RK, and T_2-RKR). Their intensities (within each series) are in fairly good agreement with those determined from HPLC data. It should be noticed that low absolute abundance of T_2 series peptides make it difficult to obtain reliable quantitative data. However, when the products of the T_2 series are the main ones in the reaction mixture, their intensities also are in good agreement with HPLC data. It could be mentioned that T_1T_2-R peak abundances are comparable with those of the principal digestion products in spite of the low T_1T_2-R concentration.

It can be concluded that the presence of basic amino acids in the sequences of analyzed peptides cannot be considered as a major factor in determining the yields of their quasimolecular ions. The "aliphatic" part of the molecule seems to be more significant in this case. These results are in good agreement with the MS analysis data of the model mixtures of amino acids and dipeptides (Table 4).

For the first mixture, the maximal intensity of quasimolecular ion yields was observed for Ile. The Arg ions were detected with slightly higher intensities than the Gly ions. At the same time, Gly and Arg intensities are sufficiently small in comparison with the Ile ones. The amino acids ions yields were compared with their gas phase proton affinities (PA)[21,22] and with Tanford's hydrophobicity indexes (ΔG),[23] describing their features in the

Table 3. Results of HPLC and ESI-MS Analysis of the Desalted Melittin Digests

	digest 1 ($t_{hydrolysis}$ = 23 min)					digest 2 ($t_{hydrolysis}$ = 3 h)			
		m/z for peptides (relative abundances)					*m/z* for peptides (relative abundances)		
		Z					*Z*		
peptide	concn from HPLC data (%)	4	3	2	total (%)	concn from HPLC data (%)	3	2	total (%)
MEL	29	3580	1390		38.5				
T_1T_2-RKR	9	832	582		10.8				
T_1T_2-RK	4	4.7	614						
T_1T_2-R	22		3607	411	30.7	>0.5	284	1158	16
T_1T_2	11		723	249	7.5				
T_2-RKR-T_3	5		477		3.6				
T_2-RKR	2								
T_2-RK	>1								
T_2-R	13		78	241	2.4	51	706	3433	47
T_2	4			237	1.8	49		3105	37

Table 4. Ratios of the Ion Yield Intensities Obtained by Mass Spectrometric Analysis of the Model Mixtures[a]

mixture	composition	$-\Delta G_{trans}^{EtOHH2O}$ (kcal/mol)	PA[14] (kcal/mol)	relative abundance of the ions (%)
1	Gly	0	211.6	100
	Ile	2.4	218.1	1000
	Arg	0.75	245.2[b]	270
2	Gly	0	211.6	100
	Ala	0.75	214.8	140
	Val	1.7	216.5	315
	Leu	2.4	216.8	370
3	Gly-Ala	0.75		100
	Gly-Val	1.5		170
	Leu-Gly	2.6		260

[a] Concentration of each amino acid and peptide was 0.5 mM. Spectra were recorded from 75% aqueous acetonitrile containing 0.1% TFA. [b] This value is from ref 15.

gas and liquid phases. Table 4 data indicate that the hydrophobicity of amino acids is the main factor that determines ion yields from the acidified solutions.

For the second mixture, the ion yields of aliphatic amino acids Gly, Ala, Val, and Leu are also found to increase with their increasing hydrophobicity. The increase in ion yields could be also explained in this case by proton affinities, but this would be in a strong disagreement with the trend in the preceding data.

Similar dependence of ion yields on hydrophobicity was observed for the series of aliphatic dipeptides Gly-Ala, Gly-Val, and Gly-Leu (Table 4). The results obtained show that (1) the ratios of intensities of the quasimolecular ions detected from amino acids and dipeptides do not coincide well with the ratios of their protonated ion concentrations in acidified solution and PA values in the gas phase; (2) the hydrophobicity index of the amino acids appeared to be the parameter showing the best correlation with quasimolecular ion yields; and (3) among MEL and its initial tryptic digestion products, the maximal ion yields are observed for peptides containing the T_1T_2 structural segments. Their relative molar concentrations chromatographically determined are in fairly good agreement with the ratios of peak intensities. The concentrations of the series of T_1 segments had not been adequately estimated by MS data since their absolute signal abundances were very low. This effect could probably be explained by their smaller hydrophobicities compared with peptides from the T_1T_2 series.

It is difficult to interpret unambiguously the dependence of the ion intensities on the amino acid compositions of the peptides. At the same time, the application of ESI-MS to the quantitative analysis of peptide mixtures seems highly attractive and stimulates efforts in this direction.

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