Volume 330, number 3, 339–342 FEBS 12933 September 1993
© 1993 Federation of European Biochemical Societies 00145793/93/$6.00

Primary structure of three cationic peptides from porcine neutrophils

Sequence determination by the combined usage of electrospray ionization mass spectrometry and Edman degradation

Olga A. Mirgorodskaya[a,*], Andrew A. Shevchenko[a], Kamal Omer M.A. Abdalla[a], Igor V. Chernushevich[b], Tsezi A. Egorov[c], Alexander X. Musoliamov[c], Vladimir N. Kokryakov[d], Olga V. Shamova[d]

[a]*Institute for Cytology of the Russian Academy of Sciences, 4 Tikhoretsky avenue, 194064 St. Petersburg, Russian Federation*
[b]*Institute for Energetic Problems of Chemical Physics of the Russian Academy of Sciences, Chernogolovka, Moscow region, Russian Federation*
[c]*Engelhardt Institute for Molecular Biology of the Russian Academy of Sciences, Moscow, Russian Federation*
[d]*Pavlov Institute for Experimental Medicine of the Russian Academy of Medicine Sciences, St. Petersburg, Russian Federation*

Received 28 June 1993

The primary structure of three major cationic peptides from porcine neutrophils has been determined. The sequencing was made by the combined use of electrospray ionization mass spectrometry and Edman degradation. The determined sequences unambiguously show that these peptides can not be considered as defensins.

Peptide from neutrophil; Primary structure; Electrospray ionization mass spectrometry

1. INTRODUCTION

The study of structural features of cationic peptides, including ones from mammalian blood neutrophils, is of particular interest due to their known antimicrobial, antifungal and antiviral activities. These properties, together with their low toxicity and absence of immunoreactivity, have led us to consider the cationic peptides from neutrophils as novel types of antibiotics [1].

In the present study, three major peptides from porcine blood neutrophils were purified and their amino acid sequences determined. The sequencing was carried out by the combined use of electrospray ionization mass spectrometry (ESI MS) and Edman degradation. The principal advantages of ESI MS are the determination of molecular masses of peptides or proteins with high accuracy, and the direct qualitative analysis of peptide mixtures without their preliminary fractionation [2,3]. The approach proposed might be especially useful for sequence determination in homologous proteins and peptides.

*Corresponding author: Fax: (7) (812) 247 0341.

Abbreviations: PNP, porcine neutrophil peptide; ESI MS, electrospray ionization mass spectrometry; HFBA, heptafluorobutyric acid; Cys*, pyridylethylcysteine residue.

2. MATERIALS AND METHODS

A freshly obtained sample of porcine blood leukocytes was homogenized in 10% acetic acid followed by centrifugation at 25,000 × *g* for 1 h. The supernatant was subjected to 24 h dialysis against 5 vols. of 10% acetic acid in Sigma 250-7U tubing. The lyophilized dialyzate was separated on a 5 × 100 cm Acrylex P 10 column equilibrated in 5% acetic acid. The low molecular weight fraction was collected and lyophilized, yielding the total porcine neutrophil peptide (PNP) preparation.

The total PNP preparation was separated by reverse-phase (RP) HPLC on a Milikhrom liquid chromatograph (Nauchpribor, Russian Federation) on 2 × 62 mm columns packed with Nucleosil 5 C_{18} (Macherey-Nagel) by a 20–35% gradient of acetonitrile in acetonitrile/0.1% TFA mixture. The eluate was detected at 210 nm.

Mass spectra were recorded on a time-of-flight (TOF) mass reflectron coupled with an electrospray ion source [4]. Samples were redissolved in 2% acetic acid/acetonitrile 1:1 (v/v), and injected at a flow rate of 2 μl/min The time of spectra registration was 0.5 min. Less than 100 pmol of the sample was required for spectra recording.

Individual peptides were reduced by β-mercaptoethanol and alkylated by 4-vinylpyridin in accordance with [5] with some modifications. The reaction mixtures were desalted by RP-HPLC. The number of cysteine residues was calculated from the difference between the masses of intact and pyridylethylated peptides.

Amino acid composition was determined using a model D-500 amino acid analyzer (Durrum). Amino acid sequences were determined using a model 816 automatic peptide sequencer (Knauer) equipped with a model 120A PTH analyzer (Applied Biosystems)

The reduced and alkylated peptides were digested by pepsin in 1% acetic acid at 37°C; the enzyme–substrate ratio was 1 : 50. Digestion products were separated by RP-HPLC.

Peptide hydrolysis was carried out by heptafluorobutyric acid (HFBA) vapors, as described in [6] with some modifications.

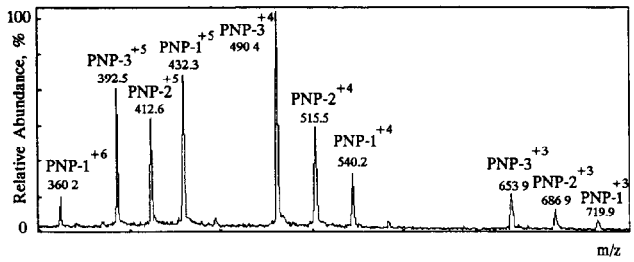


Fig. 1. Mass spectrum of the total preparation of peptides from porcine neutrophils The charges of corresponding quasimolecular ions are shown above the peak designations.

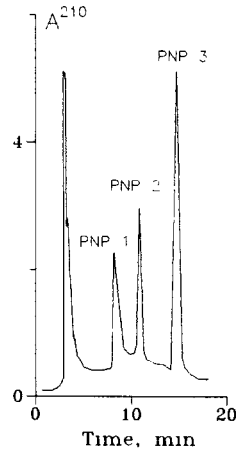


Fig. 2. Chromatogram of the total preparation of the peptides from porcine neutrophils. Experimental conditions are given in Section 2.

3. RESULTS AND DISCUSSION

The ESI mass spectrum (Fig. 1) and the chromatogram (Fig. 2) of the total PNP preparation show that it contains three major peptides (PNPs 1–3) (peptides are numbered in accordance with their elution order). Molecular masses and amino acid composition data for individual peptides are shown in Table I.

Treatment of PNPs by the Ellman's reagent showed the absence of free SH-groups. After peptide reduction followed by pyridylethylation of the formed cysteine residues, the molecular weight of each of the PNPs was increased by 424.6 Da, which shows that each of the peptides contains four cysteine residues involved in two inter-molecular disulphide bonds.

Mass spectrometric analysis of the products formed after acetylation of PNPs by the acetic anhydride/methanol mixture showed the formation of mono- and double acetyl derivatives without any methyl ester ones. As was previously found in model experiments, all free -COOH groups in peptides undergo complete esterification under these conditions (data not shown). Consequently all the PNPs have blocked C-terminals.

For mass spectrometrical mapping and sequencing, PNPs were subjected to reduction of disulphide bridges with subsequent pyridylethylation. The treated PNPs

Table I

Main characteristics for the peptides from porcine neutrophils

Peptide	M_r (Da)		Amino acid composition measured (obtained from sequence data)	Mass spectrometric mapping data	
	Measured	Calculated		M_r (Da)	Variants of amino acid composition*
PNP-1	2156.6 ± 0.5	2155.7	Gly 3.1 (3) Val 2.1 (2) Leu 1.1 (1) Tyr 0.8 (1) Phe 0.9 (1) Arg 5.8 (6) Cys_2 2 (2)	P_1 557.7	G_2R_2L, (VG_3RL)
				P_2 389.3	CY
				P_3 841.9	CR_3F, ($ZGVR_2F$, G_2V_2RCF
				P_4 844.8	V_2Z_2GR, (VR_2Z_2)
PNP-2	2057.6 ± 0.5	2056.5	Gly 4.1 (4) Val 2.1 (2) Leu 1.1 (1) Tyr 0.8 (1) Phe 1.0 (1) Arg 4.9 (5) Cys_2 2 (2)	P_1 458.5	G_3RL
				P_2 389.3	CY
				P_3 841.9	CR_3F, ($ZGVR_2F$, G_2V_2RC)
				P_4 844.8	V_2Z_2GR, (VR_2Z_2)
PNP-3	1957.5 ± 0.5	1956.5	Gly 2.1 (2) Val 1.0 (1) Leu 1.6 (1) Tyr 0.8 (1) Phe 1 0 (1) Arg 4.9 (5) Ile 0.9 (1) Cys_2 2 (2)	P_1 557.7	G_2R_2L, (VG_3RL)
				P_2 389 3	CY
				P_3 841.9	CR_3F, ($ZGVR_2F$, G_2R_2RC)
				P_4 645.6	C_2VI

*C = pyridylethylcysteine residues; alternative variants of amino acid composition are shown in parentheses.

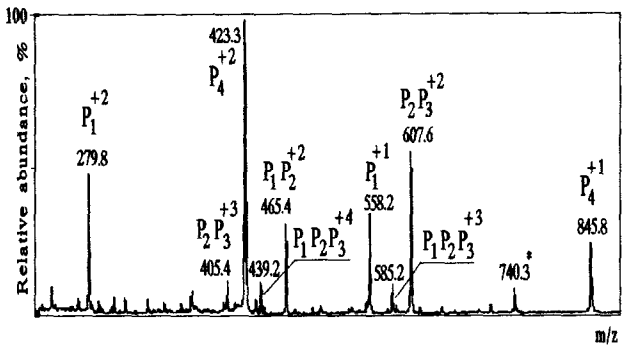
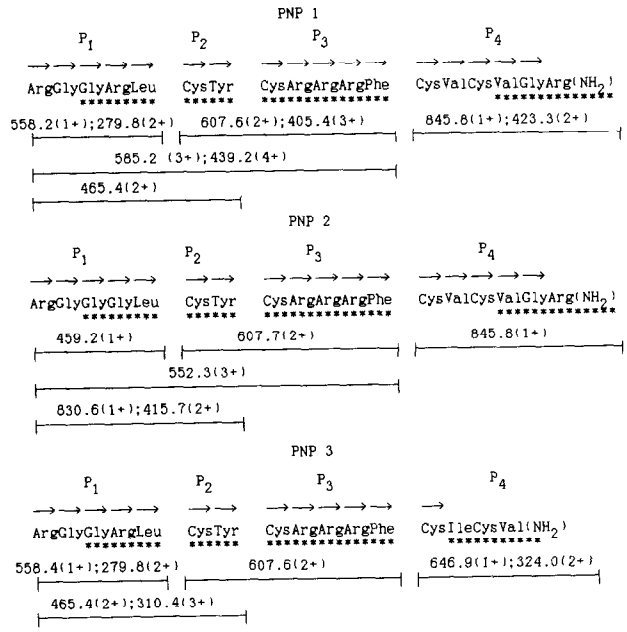


Fig. 3. Mass spectrum of the PNP 1 pepsin hydrolyzate. Ion identification is shown in Table I. The fragment ion of the P_4 quasimolecular ion formed by Cys* loss (M = 105.2) is marked by an asterisk.

were digested by pepsin. The products obtained were injected directly into the mass spectrometer. As an example, the mass spectrum of a pepsin digest of PNP 1 is presented in Fig. 3. Mass spectral analysis demonstrates clearly the presence of four structural fragments, revealed by comparison of masses of the products obtained by complete (P_1 and P_4) and partial (P_1P_2, P_2P_3, $P_1P_2P_3$) cleavage of the PNPs (Table I, Scheme I). All PNPs have two identical structural fragments (P_2 and P_3). The sequence of the P_3 fragment can only be Cys–Tyr, as follows from PNP amino acid composition and the known substrate specificity of pepsin.

The amino acid composition of fragments P_1, P_3 and P_4 can not be calculated unambigiously from their



Scheme I. Amino acid sequences of the peptides from porcine neutrophils. Note: sequences obtained by Edman degradation are shown by arrows. Sequences determined by ESI MS are shown by asterisks. Fragments identified by ESI MS mapping are shown by lines with the corresponding m/z and z values.

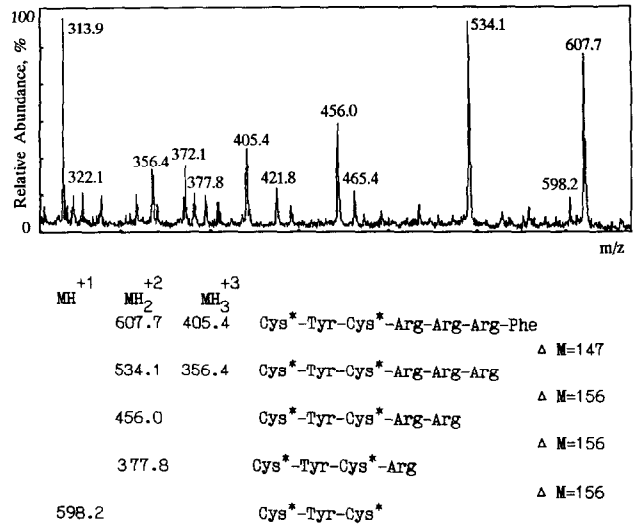


Fig. 4. Mass spectrum of the hydrolyzate of the P_2P_3 fragment of PNP 1 formed after HFBA vapor treatment.

masses alone. However, as follows from the calculation of the amino acid compositions, P_1 and P_3 fragments contain Leu and Phe residues, respectively.

Since pepsin hydrolyzes polypeptides at Leu, Phe and Tyr residues, this means that only the P_4 fragment is a C-terminal one. All masses of P_4 calculated from the amino acid composition exceed the measured values by 1 Da (Table I, Scheme I). Consequently, the C-terminals of all PNPs appear to be amidated.

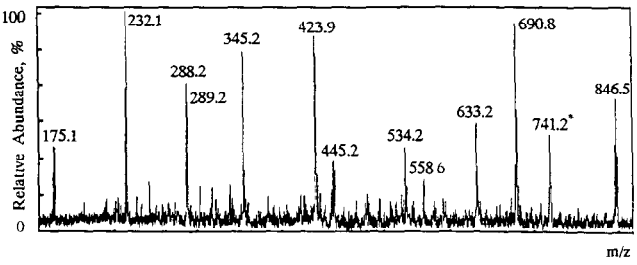
Attempts to sequence fragments P_1–P_4 were done using PNP pepsin digests separated into two fractions: a P_2P_3 fragment and a mixture of P_1 and P_4 fragments. The fractions were treated with HFBA vapor for partial C-terminal peptide hydrolysis. As an example, mass spectra of the HFBA hydrolyzates of the PNP 1 fraction and their interpretation are presented in Figs. 4 and 5. The PNP sequences determined from mass spectrometric data are shown in Scheme I.

The overlapping sequences of products obtained by partial peptic digestion of PNPs (Fig. 3 and Scheme I) allow the determination of the localization of the P_1–P_4 structural fragments in the total sequences. The data obtained show that the location of P_1–P_4 is in agreement with their numbering.

It can be seen that only the N-terminal sequences of P_1 and P_4 fragments remain ambiguous. To complete and confirm the PNP sequences, the Edman degradation technique was applied (Scheme I).

Comparison of the newly obtained sequences with the known ones of peptides from neutrophils shows that PNPs can not be considered as defensins [1]. However, PNPs and human and rabbit defensins contain some homologous sequences.

In conclusion, it should be mentioned that this study of the primary structure of homologous polypeptides revealed some advantages and disadvantages of the ESI



MH^+	MH_2^{+2}	P_4	
846.5	423.9	(Cys^*_2,Val)-Val-Gly-Arg	
690.8	345.2	(Cys^*_2,Val)-Val-Gly	Δ M=158
633.2		(Cys^*_2,Val)-Val	Δ M=57
534.2		(Cys^*_2,Val)	Δ M=99
		P_1	
558.5	279.8	(Arg,Gly)-Gly-Arg-Leu	
445.2		(Arg,Gly)-Gly-Arg	Δ M=113
289.2		(Arg,Gly)-Gly	Δ M=147
232.1		(Arg,Gly)	Δ M=57

Fig. 5. Mass spectrum of the hydrolyzate of a mixture of P_1 and P_4 fragments formed after HFBA vapor treatment The fragment ion of the P_4 quasimolecular ion formed by Cys* loss (M = 105.2) is marked by an asterisk The amino acid composition of the N-terminal parts of P_1 and P_4, calculated from their molecular masses, are shown in parentheses

MS technique. Mass spectrometric analysis of partial enzyme digests of a polypeptide chain enables one to discover the structural fragments and to determine their localization. Comparison of masses of structural fragments of homologous polypeptides allows the localization of the fragments with amino acid substitutions. Mass spectrometric analysis of peptide hydrolyzates resulting from HFBA vapor treatment enables the partial C-terminal sequences to be determined. ESI MS is a good supplement to the Edman sequencing technique, the use of which often leaves C-terminal sequences undetermined. At the same time, the ESI MS technique does not allow the determination of Leu and Ile positions in the polypeptide sequence, which must therefore be determined by other methods.

Acknowledgements: The authors are grateful to academician of Russian Academy of Sciences, D.G. Knorre, for his support of the present work and useful advice.

REFERENCES

[1] Lehrer, R.I., Ganz, T. and Selsted, M.E. (1991) Cell 64, 229–230.
[2] Alexandrov, M.L., Baram, G.I., Gall, L.N., Grachev, M.I., Knorre, V.D., Krasnov, V.N., Kusner, Y.S., Mirgorodsdkaya, O.A., Nikolaev, V.I. and Shkurov, V.A. (1985) Bioorgan. Khimia 11, 705–708.
[3] Mirgorodskaya, O.A. and Shevchenko, A.A. (1992) FEBS Lett. 307, 263–266.
[4] Dodonov, A.F., Chernushevich, I.V. and Laiko, V.V. (1992) Abstr. 12th Intern. Mass Spectrometry Conf., Amsterdam, Abstr. TUA-D20
[5] Inglis, A.S., McMahon, D.T.W., Roxburgh, C.M. and Takayanagi, H. (1976) Anal. Biochem. 72, 86–94.
[6] Tsugita, A., Takamoto, K., Kamo, M. and Iwade, H. (1992) Eur. J. Biochem 206, 691–696.