STRUCTURE OF MELITTIN ISOLATED FROM TWO SPECIES OF HONEY BEES

G. KREIL

Institut für Molekularbiologie, Österreichische Akademie der Wissenschaften, Wasagasse 9, 1090 Wien, Austria

and

Institut für Bienenkunde, Universität Frankfurt, Oberursel, Germany

Received 2 April 1973

1. Introduction

Besides the different varieties of the common honey bee, *Apis mellifera*, three additional species of the genus *Apis* can be distinguished: the Indian bee *A. cerana* and the free-nesting forms *A. dorsata* and *A. florea*. The characteristics of these species have been reviewed by Ruttner [1]. All of them possess a venom gland, which produces an aqueous secretion containing a variety of pharmacologically active compounds. Only the venom of *A. mellifera carnica*, the honey bee widely distributed in Middle Europe, has been studied in detail [2]. In particular, the amino acid sequence of melittin, the main component of this venom, has been determined by Habermann and Jentsch [3]. According to these authors, melittin is a basic peptide containing 26 amino acids arranged in a single chain.

The present report deals with the structure of melittin isolated from the venom glands of *A. cerana* and *A. florea*, respectively. While the former was found to be identical to the melittin produced by *A. mellifera*, the latter showed five differences in its sequence, which are distributed along the entire chain.

2. Materials and methods

A. cerana and *A. florea* bees are kept at the Institut für Bienenkunde (Oberursel, Germany) in the course of a research program supported by Deutsche Forschungsgemeinschaft. Bees were obtained from this institute through the generous help of Prof. F. Ruttner and Dr. N. Koeniger. Stinger, venom sac and venom gland were removed and stored at −20°C until used. The following procedure was employed for the isolation of melittin: After addition of equal volumes of 0.15 N ammonia and n-butanol (0.2 to 1 ml/100 glands), the glands were ground with quartz sand and vigorously shaken. After centrifugation, the organic layer containing the melittin could be removed; the aqueous phase was extracted two more times with the same solvent. After taking the combined butanol phases to dryness, 0.5 ml water was added. Subsequently, melittin was precipitated by addition of two volumes of saturated picric acid. Melittin-picrate was collected by centrifugation, washed twice with 70% saturated picric acid and finally cleaved by adding 2 ml acetone containing 0.2% conc. HCl. After three washes with the latter solvent, melittin was recovered as a slightly brown powder. Traces of picric acid could be removed by passing an aqueous solution of melittin, at pH about 3, over Dowex-1 in the chloride form. Melittin yields per 100 glands were about 3 mg for *A. cerana* and about 0.5 mg for *A. florea* (using this procedure, 4–8 mg of melittin can be isolated from 100 glands of *A. mellifera*).

The melittins were digested with pepsin (0.5% enzyme, w/w, pH 1.8, 35°C, 3 hr). The resulting fragments could be fractionated by paper electrophoresis at pH 4.7 (1% pyridine–acetate, 50 V/cm, 120 min, Whatman 3 MM paper). This yields two basic hepta-

Table 1
Amino acid composition of melittins.

	Number of residues*		
	A. cerana	*A. florea*	*A. mellifera***
Asp	0.11 (0)	1.07 (1) +1	0
Thr	1.76 (2)	1.85 (2)	2
Ser	0.94 (1)	0.85 (1)	1
Glu	1.90 (2)	1.14 (1) −1	2
Pro	1.09 (1)	1.08 (1)	1
Gly	2.78 (3)	3.08 (3)	3
Ala	2.00 (2)	2.00 (2)	2
Val	1.79 (2)	1.00 (1) −1	2
Met	N.D.	N.D.	0
Ile	2.69 (3)	3.88 (4) +1	3
Leu	3.79 (4)	4.11 (4)	4
Lys	3.10 (3)	4.14 (4) +1	3
His	N.D.	N.D.	0
Arg	1.88 (2)	1.07 (1) −1	2
Trp***	(1)	(1)	1
Sum	26	26	26

* Mean value from two analyses after 20 hr hydrolysis (nearest integer).
** Taken from [3].
*** The number of tryptophane residues was obtained by checking the peptic fragments with Ehrlich reagent.

N.D. = not detectable.

Table 2
Fragments characterized from *A. florea* melittin.

P1	Gly–Ile–Gly	P6T2	Asn–Lys
P2	Pro–Thr–Leu	P6T3	(Asn, Lys, Arg)
P3	Ala–Ile–Leu	P6T4	Gln-NH_2
		P6T5	(Arg, Lys)
P4	Ile–Ser–Trp	P6T6	Lys–Gln-NH_2
P5	Lys–(Thr, Gly, Ala, Val, Leu_2)	P6T7	(Arg, Lys, Gln)-NH_2
P5C1	Lys–Val–Leu	P6T8	Ile–Lys–Asp–Lys*
P5C2	Ala–Thr–Gly–Leu	P7	(Asp, Glu, Ser, Ile_2, Lys_3 Arg, Trp)
P6	Ile–(Asp, Glu, Lys_3 Arg)		
P6T1	Ile–Lys	P7T1	Ile–Ser–Trp–Ile–Lys
T1	Gly–(Gly–Ala, Ile_2, Leu) Lys		
T1P1	Gly–Ile–Gly		
T1P2	Ala–Ile–Leu–Lys		

Code used to designate peptides: P = peptic fragment, T = tryptic fragment, P C = peptic fragment further digested with chymotrypsin, etc.

* Handling of melittin at low pH (picric acid precipitation, pepsin digestion) apparently results in partial de-amidation of asparagine.

peptides and four neutral tripeptides. The latter could be resolved by paper electrophoresis at pH 1.8 (5% acetic acid adjusted with formic acid, 60 V/cm, 90–120 min). The heptapeptides were further cleaved with trypsin or chymotrypsin. Melittin can thus be degraded to a series of di-, tri- and tetrapeptides, the sequence of which was determined by Edman degradation. After each step an aliquot was removed and analyzed. Two overlapping fragments were also investigated: under the conditions used the peptic digest contains appreciable amounts of the C-terminal dekapeptide. Furthermore, from a tryptic hydrolysate (0.2 mg melittin, 10 μg trypsin, pH 8.0, 2 hr, 37°C), the N-terminal heptapeptide could be isolated by paper electrophoresis at pH 4.7.

The N-terminal residue of both melittin was determined according to Gray and Hartley [4]. The intact toxin and its fragments were hydrolyzed in 6 N HCl containing 0.2% thioglycollic acid at 105°C for 15 hr. A Beckman analyzer was used for the quantitative analysis. For the qualitative analysis of smaller peptides, paper chromatography was employed (n-butanol/

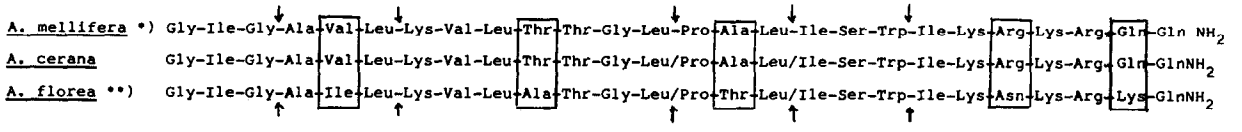

Fig. 1. Amino acid sequence of melittin from three species of honey bees. Arrows mark the bonds cleaved by pepsin. * Taken from [3]. ** / indicates that no overlapping peptide has been analyzed in this region.

acetic acid/water = 4/1/5). The net charge of the basic fragments could be deduced from the electrophoretic mobility relative to free lysine.

3. Results and discussion

The amino acid composition of melittin from *A. cerana* and *A. florea* is shown in table 1. The former was found to have the same composition as the melittin from *A. mellifera,* while that of *A. florea* contains one extra residue each of Ile, Lys and Asp (the latter residue is not found in the other melittins) and lacks one Val, Arg and Glu. The data presented in fig. 1 also show that the three melittins have the same chain length, each containing 26 residues. Glycine was found to be the sole N-terminal residue in all cases.

The structure of melittin from two species of honey bees was mainly derived from an analysis of the peptic fragments. Table 2 shows the results obtained with *A. florea* melittin. The same type of investigation was performed for the melittin of *A. cerana,* but in all cases the fragments were identical to the ones obtained from *A. mellifera* melittin [3]. It is therefore concluded that these two melittins have the same amino acid sequence.

The fragments isolated from the enzymatic digests of *A. florea* melittin can unambiguously be ordered along the known sequence of melittin from *A. mellifera* (fig. 1). From the data shown in the table 2, the sequence of *A. florea* melittin can by itself largely be deduced, since overlaps are missing only for the position of the tripeptide Pro–Thr–Leu. The comparison in fig. 1 shows that melittin from *A. florea* differs in 5 positions from that of *A. cerana* and *A. mellifera.* If the universality of the genetic code, as determined for *E. coli,* is accepted, all but one of observed differences can be the result of a single change in the first base of the respective codons. Only the replacement of asparagine in *A. florea* melittin by arginine in the higher forms requires at least two point mutations. The observed differences between these melittins probably do not lead to any drastic changes in the architecture of the molecule. In fact, several characteristic features, such as the cluster of basic amino acids near the C-terminus, the absence of a free carboxyl group from the entire molecule and the distribution of residues with hydrophobic side chains, are all conserved. Still, it will be interesting to test whether the differences found in *A. florea* melittin lead to any changes in its pharmacological properties.

This comparative investigation on the structure of melittin from different species of honey bees falls in line with morphological and other criteria. *A. mellifera* and *A. cerana* are closely related to each other. Cross fertilization is still possible, but development of the hybrid embryo stops at an early stage [5]. Apparently, in the evolution of honey bees, these two species diverged only recently and it is therefore not surprising, that their melittins are identical. On the other hand, *A. florea* is a more distant relative, which has only half the number of chromosomes (n = 8), is much smaller than the other bees (about the size of a housefly) and shows many distinct features [1]. This is borne out at the molecular level as in a peptide containing only 26 residues a total of five differences could be detected. The basic mechanism involved in the production of melittin, however, has apparently not changed during the evolution of honey bees. For *A. mellifera,* it has been demonstrated that in the venom gland, a precursor termed promelittin is synthesized first, from which melittin is later liberated through proteolytic cleavage [6, 7]. In two experiments, *A. florea* bees received radioactive amino acids ([^{3}H]Ile and [^{14}C]Leu) in their drinking water. From the venom glands of these bees, a labelled peptide could be isolated, which showed all the characteristics of promelittin as analyzed from *A. mellifera.*

Acknowledgements

This work would not have been possible without the help of Prof. Dr. F. Ruttner and Dr. N. Koeniger, who supplied me with honey bees. Mr. Paukovits kindly performed numerous amino acid analyses.

References

[1] F. Ruttner, in: Traité de biologie des abeilles, ed. R. Chauvin (Masson et Cie, Paris, 1968) Vol. 1, p. 2.
[2] E. Habermann, Science 177 (1972) 314.
[3] E. Habermann and J. Jentsch, Z. Physiol. Chem. 348 (1967) 37.

[4] W.R. Gray and B.S. Hartley, Biochem. J. 89 (1963) 379.
[5] F. Ruttner and V. Maul, Transct. XXI Beekeeping Congress, Munich (1969) pp. 510, 561.
[6] G. Kreil and H. Bachmayer, European J. Biochem. 20 (1972) 344.
[7] G. Kreil, European J. Biochem. 33 (1973) 558.