THE STRUCTURE OF *APIS DORSATA* MELITTIN: PHYLOGENETIC RELATIONSHIPS BETWEEN HONEYBEES AS DEDUCED FROM SEQUENCE DATA

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1. Introduction

Melittin, the main component of bee venom, has been characterized from three of the four species belonging to the genus *Apis*. The amino acid sequence of the melittins synthesized by *A. mellifera*, the common European honeybee, and *A. cerana* was found to be identical, while in the sequence of *A. florea* melittin, 5 out of a total of 26 residues were found to be different [1,2]. In this report, I present the structure of melittin isolated from venom glands of the fourth species, *A. dorsata*.

2. Methods and results

Apis dorsata bees imported from Ceylon are kept at the Institut für Bienenkunde (University of Frankfurt, GFR) in continuation of a research program supported by the Deutsche Forschungsgemeinschaft. The sting including venom sac and venom gland were removed from the insects, immediately frozen and subsequently lyophilized. Dried tissue from about 200 bees was kindly put at my disposal. This was suspended in 1 ml *n*-butanol and 1 ml 0.1 M ammonia and melittin was prepared as described for the other species [2]. Contrary to the other melittins, the *dorsata* peptide was found to precipitate only slowly from an icecold solution of 2/3 saturated picric acid. Over a period of 24 hr, five portions of melittin picrate were collected by centrifugation. The total yield was about 0.5 mg (approx. 0.17 μmol) of peptide. This is less than 10% of the amount which can be isolated from a similar number of *A. mellifera* bees, even though the latter have smaller venom glands. About one half of the *dorsata* melittin was used for preparing a peptic digest, one fifth for amino acid analysis, and the remainder for a tryptic digest and an end-group determination.

Amino acid analysis yielded the following composition for the *A. dorsata* melittin: Thr_1, Ser_2, Glu_2, Pro_1, Gly_3, Ala_2, Val_1, Ile_4, Leu_4, Lys_3, Arg_2. A tryptophane residue was detected in one of the peptic fragments with Ehrlich's reagent. This analysis shows that like the other melittins the *dorsata* peptide is also comprised of 26 residues. Compared to *mellifera* melittin it contains an extra residue each of serine and isoleucine and one less of threonine and valine. Using the method of Gray and Hartley [3], glycine was found to be the only N-terminal residue.

Enzymatic digests of the *dorsata* melittin were prepared and fractionated as described for the other melittins [2]. Hydrolysis with pepsin yielded 4 neutral tripeptides and 2 basic heptapeptides, small amounts of a dekapeptide derived from the carboxyl end were also detected. From the tryptic digest only the fragments liberated from the terminal regions were analyzed. The amino acid sequence of the different fragments is shown in table 1. The C-terminal structure

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Table 1
Fragments analyzed from *A. dorsata* melittin

P1	Gly–Ile–Gly	P6T1	Lys–Arg
P2	Pro–Ala–Leu	P6T2	Ile–Lys–Arg
P3	Ala–Ile–Leu	P6T3	Arg
P4	Ile–Ser–Trp	P6T4	Ile–Lys
P5	Lys–(Thr,Ser,Gly,Val,Leu_2)	P6T5	(Arg,Glu_2)
P5C1	Lys–Val–Leu	P6T6	Gln–Glu–NH_2
P5C2	Ser–Thr–Gly–Leu	P7	(Ser,Glu_2,Ile_2,Trp,
P6	Ile–(Lys_2,Arg_2,Glu_2)		Lys_2,Arg_2)
		P7T1	Ile–Ser–Trp–Ile–Lys
T1	Gly–(Ile,Gly)–(Ala,Ile)–Leu–Lys		
T2	Gln–Glu–NH_2		

Code used for the fragments: P = peptic fragment, T = tryptic fragment, PC = peptic fragment further digested with chymotrypsin, etc.

of this melittin deserves some comment. Fragments P6T6 and T2 both yield only glutamic acid after total hydrolysis and this amino acid is also N-terminal. Upon paper electrophoresis at pH 4.8, these fragments migrate slowly to the cathode (mobility about 0.12 relative to free lysine). They are therefore neither glutaminyl-glutamine, which is neutral, nor glutaminyl-glutaminyl-amide, the corresponding fragment from *mellifera* melittin, which migrates further to the cathode. After one step of Edman degradation, fragments P6T6 and T2 both yield a compound indistinguishable from iso-glutamine (α-glutamyl-amide). This was established by co-electrophoresis with authentic iso-glutamine at pH 4.4 and 1.8. This demonstrates that the fragments P6T6 and T2 have the sequence glutaminyl-isoglutamine, abbreviated as Gln-Glu-NH_2.

In case of *mellifera* melittin it has been observed that the three amide groups present in the C-terminal Gln-Gln-NH_2 sequence are slowly hydrolyzed at acid pH and each of the three possible isomers with one free carboxyl group is formed in about equal yield (unpublished experiments). Such a slow and random hydrolysis does, however, not explain the results obtained with the *dorsata* melittin, since only Gln-Glu-NH_2 but none of the other isomers could be detected in the digests. The available evidence thus show that contrary to all the other melittins the one isolated from *A. dorsata* contains one free carboxyl group. This negative charge close to the cluster of basic amino acids may impair the formation of a picrate and thus explain the slow and probably incomplete precipitation of *dorsata* melittin with picric acid.

From the structure of the fragments shown in table 1, the amino acid sequence of *A. dorsata* melittin can be deduced. This sequence is shown in fig.1. At two points, no overlapping fragment has been analyzed, but based on the known terminal sequences, alignment of the fragment is unambiguous. In table 2 the differences between the melittins of the four honeybee species are summarized.

Gly–Ile–Gly–Ala–Ile–Leu–Lys–Val–Leu–Ser–Thr–Gly–Leu/Pro–Ala–Leu/Ile–Ser–Trp–Ile–Lys–Arg–Lys–Arg–Gln–Glu–NH_2

Fig.1. Amino acid sequence of melittin from *A. dorsata*. Residues common to all melittins are underlined. A bar (/) indicates that no overlapping peptide has been analyzed in this region.

Table 2
Variant residues in melittins from different species

Species	Number of residue					
	5	10	15	22	25	26
A. mellifera *A. cerana*	Val	Thr	Ala	Arg	Gln	Gln
A. dorsata	Ile	Ser	Ala	Arg	Gln	Glu
A. florea	Ile	Ala	Thr	Asn	Lys	Gln

3. Discussion

It has been established that two of the four species belonging to the family of honeybees, namely *A. mellifera* and *A. cerana*, are closely related [4]. Cross fertilization between these bees is still possible, but embryonic development stops at an early stage [5]. This close relationship is borne out at the molecular level since it was found that these two species synthesize melittins of identical structure. On the other hand, the relationship between the *mellifera-cerana* pair and *A. dorsata* and *A. florea*, respectively, is poorly understood. A comparison of the melittins from these species shows that the *dorsata* peptide differs by five residues from the *florea*, but only by three residues from the other two peptides. At the nucleic acid level the remote relationship of *A. florea* to the other species is even more evident: a minimum of six base changes are required in the corresponding messenger RNAs to convert the *florea* to either the *dorsata* or the *mellifera/cerana* sequence (using the genetic code as determined for *E. coli*), while only three nucleotides have to be replaced to go from the *dorsata* to the latter sequence. Thus, while *A. dorsata* still has the same number of chromosomes as *A. florea* (n=8 as opposed to n=16 for the other two species), its melittin is already more similar to that of *A. mellifera*. Based on the structure of melittin, a phylogenetic tree can thus be proposed for the genus *Apis*, where the line of descent for *A. florea* branches off first from the trunk representing the ancestor common to all honeybees. Recent studies by Engels et al [6–8] on the electrophoretic mobility and immunological cross-reactivity of hemolymph proteins from the different species have independently led to an identical picture of the evolutionary history of honeybees.

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