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Original article

Enzymatic equipment of *Ascosphaera apis* and the development of infection by this fungus in *Apis mellifera*

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Summary — The process of infection of honey bee brood by the fungus *Ascosphaera apis* was initiated by feeding 3rd instar larvae with a known dose of spores. These larvae were extracted from their sealed cells at 2 different times during early postcapping development and processed for SEM. This study was complemented with an analysis of the enzymatic equipment of the fungus, to investigate the mechanism of penetration of the larval cuticle. This analysis was conducted on mycelium isolated from mummies produced in 47 field outbreaks of chalkbrood and on 3 reference strains. There was evidence of enzymes such as *N*-acetyl-β-glucosaminidase in 74.4% of the isolates, which can break down the molecules of *N*-acetyl-glucosamine. Chitin, the main component of the cuticle, is a polymer composed of these molecules. This enzyme, assisted by a certain pressure from the hyphae, as these fungal elements are generated inside the larval body, may explain how the fungus pierces the cuticle.

***Ascosphaera apis* / *Apis mellifera* / larval cuticle / penetration / infection / enzyme**

INTRODUCTION

The fungus *Ascosphaera apis* (Maassen ex Claussen) Olive and Spiltoir, is the causative agent of chalkbrood disease in the honey bee, *Apis mellifera*. Diseased larvae are covered by a fluffy white mould, becoming mummies when the invasion of the fungus is complete. Several histological studies have been made on larvae which developed infection by the fungus (Carrera *et al*, 1987; Bamford and Heath, 1989a). Nevertheless, there is controversy concerning the route of infection by *A apis*

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in the larvae, based on the evidence of penetration of the brood cuticle without production of a chitinase (Heath, 1982). Further elucidation of the mechanism of penetration during the process of infection by this fungus is needed.

MATERIAL AND METHODS

Processing infected larvae for SEM

A single dose of 5 x 10^5 spores was administered to 3rd instar honey bee larvae. A homogeneous suspension of free spores in sterile water was obtained from 15 black mummies (Gilliam *et al,* 1988), mixed with honey (50% w/v) and fed to larvae. Concentration of spores was checked using a hemocytometer, and consumption by the larvae was confirmed by direct observation.

Pieces of comb containing groups of 22–50 individuals were kept in a commercial hive after the brood received the spores until the sealing of cells, and then transferred to an incubator, where they were kept under appropriate conditions for development (30°C, 70–80% relative humidity). This brood received a cooling (22 ± 2°C) during the 24 h following operculation. Cells were opened 36 and 51 h after sealing. At least 10 larvae in each group were fixed in 4% glutaraldehyde in 0.2 M phosphate buffer and post-fixed in 1% OsO_4 in the same buffer. Specimens were dehydratated with a graded series of ethanol and dried in a critical-point drier. A Philips 501-B SEM microscope was used.

Enzymatic equipment of A apis

Source of *A apis*

Three reference strains of this fungus were used: strain number 1187 (Risö National Laboratory, DK); strains NHL 2714 and SANK 11479 (National Institute of Hygienic Sciences, Tokyo, Japan). Mummies were collected from 47 field outbreaks of chalkbrood, produced in the same number of geographical localizations in Spain. From this material, 125 non-sporulated isolates were obtained by culture in MY20 agar (Takatory and Tanaka, 1982), with 0.1% cloramphenicol added. Enzymatic studies of the reference strains were made on 2 non-sporulated isolates of each. Aseptic techniques were followed during the entire process. Identification of *A apis* in the cultured plates was tested by:

– cross-compatibility tests with reference strains (Christensen and Gilliam, 1983);

– a morphological study of the 3 characteristic reproductive structures of the genus *Ascosphaera* (Von Arx, 1981; Alexopoulos and Mims, 1985) based on the measures proposed by Skou (1972). This study was made on the sporulated cultures obtained in each compatibility test.

Enzymatic study

The presence of 19 enzymatic activities were studied using the following methods.

Qualitative methods

Urease (Christensen, 1946), phosphatase, (Hankin and Anagnostakis, 1975; Cabañes *et al,* 1988), catalase, oxidase (Cowan and Steel, 1974; Cabañes *et al,* 1988) lipolytic activity (Hankin and Anagnostakis, 1975; Crippa *et al,* 1987), lecitinase (Mossel *et al,* 1967), DNAase (Hankin and Anagnostakis, 1975), RNAase (Jeffries *et al,* 1957; Hankin and Anagnostakis, 1975; Cabañes *et al,* 1988), gelatinase (Hankin and Anagnostakis, 1975; Mac Faddin, 1980), caseinolytic activity (Ahearn *et al,* 1968), elastase (Kothary *et al,* 1984), pectate lyase (Hankin *et al,* 1971), polygalacturonase (Van der Walt and Yarrow, 1984), amylolytic activity (Hankin and Anagnostakis, 1975; Hendrickson, 1987), cellulolytic activity (Cabañes *et al,* 1988) and chitinase (Hankin and Anagnostakis, 1975; Crippa *et al,* 1987); see Alonso (1991) for more details about the methods (table I).

The semiquantitative method of API ZYM^R (BioMérieux) (Gilliam et al, *1989)*

In this last method, 5 nmol of hydrolyzed substrate must be produced to consider the reaction positive. Sabouraud glucose agar was used for this test over a 7-d period at 30°C. With both methods, each test was repeated twice on every isolate (table II).

Table I. Enzymatic activities detected by qualitative methods in 3 reference strains (RS) of *A apis* and in 125 nonsporulated isolates from 47 field outbreaks of chalkbrood.

Enzymatic activity	*Positive isolates from field outbreaks (%)*	*Presence in RS*		
		RS1	*RS2*	*RS3*
Urease	0.0	–	–	–
Phosphatase	100.0	+	+	+
Catalase	100.0	+	+	+
Oxidase	0.0	–	–	–
Lipolytic on Tween 20	0.0	–	–	–
Lipolytic on Tween 80	0.0	–	–	–
Lecitinase on SA	0.0	–	–	–
Lecitinase on NA	0.0	–	–	–
DNAase	100.0	+	+	+
RNAase	100.0	+	+	+
Gelatinase in plate	98.4	+	+	+
Gelatinase in tube	98.4	+	+	+
Caseinolytic	100.0	+	+	+
Elastase	0.0	–	–	–
Pectate lyase (pH 7)	0.0	–	–	–
Polygalacturonase (pH 5)	0.0	–	–	–
Amylolytic	100.0	+	+	+
Cellulolytic	100.0	+	+	+
Glycogenolytic	100.0	+	+	+
Chitinase	0.0	–	–	–

RS1: *A apis* 1187 (Denmark); RS2: *A apis* NHL 2714; RS3: *A apis* SANK 11479 (Japan); SA: Sabouraud agar; NA: nutritive agar.

RESULTS AND DISCUSSION

The image presented in figure 1 was seen in all the inoculated larvae. The surface of a honey bee larva (S) is locally raised by the fungal elements generated from inside the larval body (arrows). One of the hyphae (H) has pierced the cuticle. The possible participation of a pressure mechanism in the piercing of the larval cuticle has been pointed out by Carrera *et al* (1987) *via* optic microscopy. The image presented in figure 1 may confirm this action. Results of enzymatic activity are presented in tables I and II.

Qualitative methods show a lack of variability in the enzymatic activity of isolates

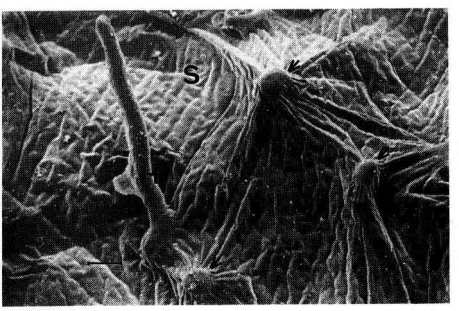

Fig 1. SEM image of the hyphae of *A apis* piercing the larval cuticle in a honeybee prepupa. This larva received fungal spores at the 3rd instar. Bar: 5 μm. S: surface of the larvae; H: hypha piercing the cuticle; arrows: hyphae raising regions of the cuticle before piercing it.

Table II. Enzymatic activity detected by the API ZYM^R (BioMérieux) semiquantitative method in the same isolate as in table I.

Enzymatic activity	*Positive isolates from field outbreaks (%)*	*Presence in RS*		
		RS1	*RS2*	*RS3*
Alkaline phosphatase	100.0	+	+	+
Acid phosphatase	100.0	+	+	+
N-Acetyl-β-glucosaminidase	74.4	–	+	+
Esterase (C4)	100.0	+	+	+
Esterase-lipase (C8)	98.4	+	+	+
Lipase (C14)	0.0	–	–	–
Leucine aminopeptidase	100.0	+	+	+
Valine aminopeptidase	98.4	+	+	+
Cystine aminopeptidase	18.4	–	–	–
Trypsin	0.0	–	–	–
Chymotrypsin	21.6	–	–	–
Phosphoamidase	100.0	+	+	+
α-Galactosidase	0.0	–	–	–
β-Galactosidase	84.8	–	+	+
β-Glucuronidase	0.0	+	+	+
α-Glucosidase	80.0	+	+	+
β-Glucosidase	99.2	+	+	+
α-Mannosidase	95.2	+	+	+
α-Fucosidase	0.0	–	–	–

(table I). This homogeneity was also detected by Glinski and Osipowski (1987) and Vey (1990), and can be attributed to the seasonal migration of hives in Spain, which maintains the contact between apiaries of very diverse origin.

Catalase and phosphatase activity detected in all our isolates were previously found by Chmielewski and Glinski(1981) and Gochnauer and Margetts (1979) respectively almost in the same percentage. On the other hand, the latter authors failed to detect DNAase and RNAase activity, which we found in all our isolates, using the same method (Hankin and Anàgnostakis, 1975). With respect to protein substrates, gelatinase activity was present in 98.4% of the isolates and caseinolytic activity was present in 100%. In the study by Chmielewski and Glinski (1981), only 58.7 and 1.8% of the isolations respectively, were found to be positive. Gochnauer and Margetts (1979) also failed to demonstrate a marked protease activity of the fungus, and concluded that *A apis* is a relatively non-invasive parasite that kills the host by competition for primary nutrients.

Concerning the enzymatic action on carbohydrate substrates, the fungus seems to be more active than has previously been described. Amylolytic activity, which was not detected for Gochnauer and Margetts (1979) or only detected in low percentages by Chmielewski and Glinski (1981), was present in all of our isolates. Cellulolytic enzyme activity which had previously been unreported, was present in 100% of the samples. Glycogenolytic activity, described in a low proportion of samples by Kowalska (1984), was also found in 100% of the isolates, a percentage similar to that

(91.7%) described for this activity by Chmielewski and Glinski (1981).

Our results also confirm the lack of chitinase production by *A apis* (Huber, 1958; Gochnauer and Margetts,1979; Chmielewski and Glinski, 1981; Kowalska, 1984). This is important in relation to the mechanism of penetration of the larval cuticle by the fungus.

Results presented in table II confirm the presence of phosphatase activity, both alkaline and acid phosphatase, in 100% of the isolates.

N-acetyl-β-glucosaminidase activity was present in 74.4% of the isolates. A study by Gochnauer and Margetts (1979) failed to demonstrate the presence of chitinase and other crucial lytic enzymes; Bamford and Heath (1987) suggested that bound enzymes may be involved in the mechanism of cuticular penetration by the fungus. The mechanism of penetration of the larval cuticle by the fungus might be explained by a combined action, a selective attack on the monomers of *N*-acetyl-β-glucosamine which composes the chitin, and a pressure mechanism by the hyphae generated from below the cuticle, as described before. Insect cuticle is composed of lipids and proteins in addition to chitin (Richard and Davies, 1983; Boucias and Latge, 1988). The presence of lipolytic and proteolytic enzymatic activities (esterase, esterase-lipase, leucine and valine aminopeptidase) found by the API ZYM^R method in almost all the isolates, may be complemented by the action on proteic substrates found by qualitative methods.

The API ZYM^R system also confirmed that *A apis* shows extensive enzymatic equipment for the degradation of carbohydrates. A high percentage of isolates produced β-galactosidase, α-glucosidase, β-glucosidase, and α-mannosidase.

The percentage of isolates found to be positive for various enzymatic activities coincides with that detected in the reference strains, both by the qualitative as well as by the semiquantitative method (tables I, II).

By the use of these 2 methods for enzyme analyses, it can be seen that *A apis* develops an extensive enzymatic activity in the main components of the cuticle. Infection from ingested material would probably lead to a complementary mechanism for piercing of the larval cuticle, supported by the pressure produced by hyphae when these elements are generated from inside the larval body. If the fungus can proliferate from the surface of the brood, as has been pointed out by several authors (Gilliam *et al*, 1978; Glinski, 1981), enzymes would have to carry out the piercing of the cuticle without internal hyphal pressure contribution.

A apis appears to have a life-cycle which is highly adapted to the development of the honeybee brood. Bamford and Heath (1989b), in a study of the effects of environmental temperature and pH on the germination of *A apis*, have pointed out that the fungus is better adapted to germinate within a larva than on its surface. The wide range of mechanisms for degradation of carbohydrates would contribute to rapid growth inside the larval gut contents. The stage at which penetration of the gut wall usually takes place coincides with the destruction of the midgut cells, providing hyphae with an easy route thoughout the larval body (Heath, 1982). By the time the hyphae reach the surface cuticle, the bee larva is dead, so its own protolytic and other enzymes could contribute to cuticle breakdown. Besides, when the fungus reaches this cuticle, its hyphae have grown sufficiently to develop a strong pressure on this structure and, leaning on the underlying tissues, the proteolytic, lipolytic and *N*-acetyl-β-glucosaminidase action combine with this mechanical effect. All these circumstances would not be present in the case of surface infections.

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Résumé — L'équipement enzymatique d'*Ascosphaera apis* et le développement de l'infection chez l'abeille, *Apis mellifera*. On déclenche l'infection du couvain par le champignon *Ascosphaera apis* en administrant à des larves de 3e stade une dose connue de spores. Des larves ont été extraites de leur cellule operculée 36 h ou 51 h après l'operculation, et traitées pour être observées au microscope électronique à balayage. Cette étude est complétée par une analyse de l'équipement enzymatique du champignon, afin de comprendre le mécanisme de pénétration de la cuticule larvaire. L'analyse a été faite sur des myceliums obtenus à partir de momies prélevées dans 47 foyers de couvain plâtré, et sur 3 souches de référence, selon des méthodes qualitatives et semi-quantitatives (API ZYM^R, BioMérieux) (tableau I et II) Bien qu'on n'ait pas détecté d'activité nettement chitininasique dans les isolements, on note la présence évidente, dans une partie des échantillons analysés, d'enzymes tels que l'acétyl glucosaminidase. Cet enzyme peut attaquer les monomères de N-acétyl glucosamine qui composent la chitine, principal composant de la cuticule. Cette action enzymatique, aidée d'une certaine pression mécanique des hyphae quand ces éléments fongiques sont générés depuis l'intérieur du corps larvaire (fig 1), peut expliquer comment le champignon traverse la cuticule. La présence d'un vaste arsenal enzymatique destiné à la dégradation des sucres confirme que *A apis* est un agent pathogène des larves d'abeille hautement spécialisé.

***Ascosphaera apis* / *Apis mellifera* / cuticule larvaire / pénétration / infection / enzyme**

Zusammenfassung — Enzymatische Ausstattung von *Ascosphaera apis* und Entwicklung der von diesem Pilz hervorgerufenen Infektion bei *Apis mellifera*. Der Infektionsablauf wurde hervorgerufen, indem man Larven im 3.Stadium eine bekannte Dosis von Sporen des Pilzes *Ascosphaera apis* eingab. Die für das REM präparierten Larven stammten aus verdeckelten Zellen, die man zu zwei verschiedenen Zeitpunkten entnahm (36 und 51 Stunden nach Verdeckelung der Zellen). Diese Studie wurde durch eine Analyse des enzymatischen Arsenals des Pilzes vervollständigt, um den Penetrationsmechanismus durch die Kutikula der Larve aufzuklären. Die Untersuchung wurde mit aus Mumien isolierten Myzelien durchgeführt,welche aus 47 Epizootien der Kalkbrut und 3 Stämmen als Referenz stammen, wobei qualitative und halbquantitative Methoden (API ZYM^R, BioMérieux) angewandt wurden (Tabelle I und II). Obwohl in keiner der isolierten Proben Enzyme nachgewiesen wurden, die ausschließlich das Chitin angreifen, ist es offensichtlich, daß 74,4% der isolierten Proben Enzyme wie die Acetylglukosaminase enthalten, die die Kette des Chitins aufbrechen können. Das Chitin ist ein Polymer, das sich aus N-Acetylglukosaminmolekülen zusammensetzt und ein Hauptbestandteil der Kutikula bildet. Diese enzymatische Wirkung, zusammen mit dem mechanischen Druck, den die

Hyphen aus dem Inneren der Larve herauswachsend ausüben (Abb 1), kann die Penetration der Kutikula durch den Pilz erklären. Das Vorhandensein eines umfassenden enzymatischen Arsenals für den Abbau von Zuckern bestätigt, daß *Ascosphaera apis* einen hochspezialisierten Krankheitserreger der Bienenlarve darstellt.

***Ascosphaera apis* / *Apis mellifera* / Larvenkutikula / Durchdringung / Infektion / Enzyme**

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