Effect of cuticular compounds on the elastase activity of the entomapathogenic fungus *Conidiobolus coronatus*

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BACKGROUND

Entomopathogenic fungi are important natural regulatory factors of insect populations. They invade insects through the hard cuticle by a combination of mechanic pressure and enzymatic degradation. Insecticidal fungi produce several cuticle degrading proteases, chitinases and lipases. Due to the 70% participation of proteins in the construction of insect cuticle, it is believed that proteases play the greatest part in the degradation of cuticle while roles of chitinases and lipases are minor. Among proteases produced by soil fungus *Conidiobolus coronatus* (Entomophtorales) **(Figures 1, 2)**, elastase seems to play a key role in the hydrolysis of cuticle.

The process of infection is initiated by the germination of fungal spore **(Figure 3)** and thanks to mechanical pressure of growing hyphae and cuticle degrading enzymes hyphae of *C. coronatus* reach victim's hemocoel. *C. coronatus* kills susceptible insect species rapidly and efficiently while species resistant remain unhurt. Attacked *Galleria mellonella* larvae die within 1-2 days after contact with *C. coronatus* (symptoms of infection: melanotic spots on the cuticle, immobilization of larvae) **(Figures 3, 4)**.

Early work based on histological studies showed that in insects susceptible to infection **(Table 1)** *C. coronatus* hydrolyzes cuticle, as indicated by the presence of hyphae in the *Dendrolimus pini* hemocoel **(Figure 5a).** Contact with fungal hyphae activates the immunological response of *G. mellonella* hemocytes **(Figure 5b)** which are forming melanotic capsule surrounding pathogen. In contrast, fungal spores cannot germinate on the cuticle of *Calliphora vicina* larvae which are resistant to fungal infection **(Figure 5c).**

Although mechanisms of enzymatic degradation of cuticle are intensively studied, the reasons of differential susceptibility of various insect species to fungal infection remain obscure. Hypothesis that susceptibility or resistance of various insect species to fungal invasion may result from the species-specific composition of the cuticle is strongly supported by the observation that cuticular fatty acids as well as various C and N sources affect growth and pathogenicity of *C. coronatus* [2, Włóka, unpublished data].

Figure 1. *C. coronatus* on Sabouraud agar medium

Figure 2. *C. coronatus* on Sabouraud agar medium enriched with homogenized *G. mellonella* larvae

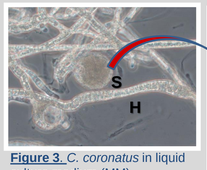


Figure 3. *C. coronatus* in liquid culture medium (MM)

S-spore, **H**-hyphae

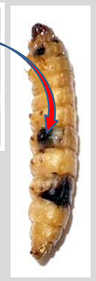


Figure 4. Infection of *G.mellonella* larvae (Lepidoptera) with fungus *C. coronatus*

1- intact larvae
2 to 6 – process of infection

Mortality of *G. mellonella* larvae **80-100%** within 1-2 days of contact with *C. coronatus*

Table 1. Various sensitivity of 3 insect species (larvae) to *C. coronatus* [1]

Insect species	*Dendrolimus pini*	*Galleria mellonella*	*Calliphora vicina*
No. of insect used	33	112	54
% of mortality ± SD	88 ± 6	92 ± 1	0

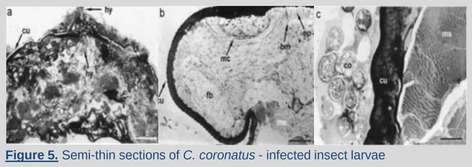


Figure 5. Semi-thin sections of *C. coronatus* - infected insect larvae

bm - basement membrane, **cu** – cuticle, **co** - fungal conidia, **ep** – epidermis, **fb** - fat body, **hy** - fungal hyphae, **mc** - melanotic capsule, **ms** - muscles
Scale bars - 100 µm [1]

OBJECTIVES

The aim of the study was to examine effects of compounds detected in insect cuticle and various C and N sources on the activity of *C. coronatus* elastase.

MATERIALS AND METHODS

•Culture conditions

The *C. coronatus* isolate 3747I-$_1$ was propagated in minimal liquid medium (MM) (20^{o}C, 3 weeks). In biochemical studies post-culture filtrates were used.

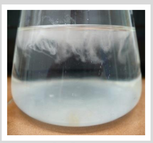


Figure 6. *C. coronatus* in liqiud medium MM

•Protein assay

Total protein content was estimated using bovine serum albumin as standard [3].

• Elastase activity assay

The elastolytic activity of the post-incubation filtrates was determined using chromogenic substrate N-Succinyl-Ala-Ala-Pro-Phe-pNA (Sigma) as described previously [4]. Effects of MM supplementation with compounds previously detected in the cuticle of various insect species [5, 6] on the activity of *C. coronatus* elastase was assayed using 11 fatty alcohols **(Figure 7)**, 16 fatty acids **(Figure 8)**, butyl oleate, butyl stearate, glycerol oleate, squalene, and tocpoherol acetate **(Figure 9)**. MM was supplemented with peptone, yeast extract, sucrose, elastin, chitin, N-acetylglucosamine, and *G. mellonella* larvae cuticle as well **(Figure 10)**.

•Proteolytic activity assay

Total amount of proteases was estimated using Protease Fluorescent Detection Kit (Sigma). Protease activity was assayed in MM post-incubation filtrates supplemented with peptone, yeast extract, sucrose, elastin, chitin, N-acetylglucosamine, and *G. mellonella* larvae cuticle according to the manufacturer's manual.

RESULTS

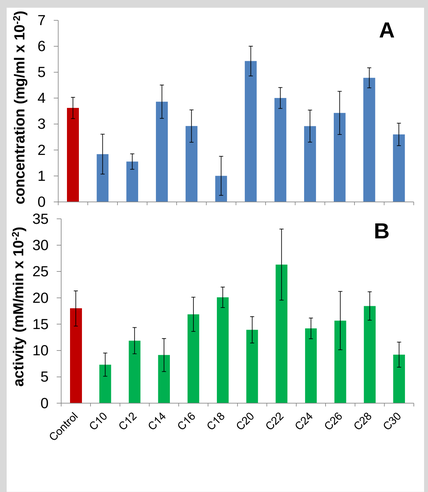


Figure 7. The effect of cuticular fatty alcohols on protein concentration **(A)** and *C. coronatus* elastase activity **(B)**

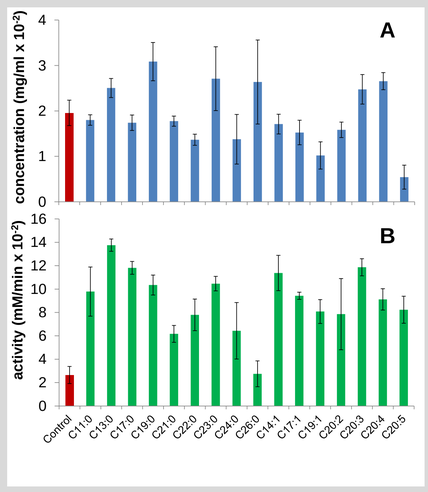
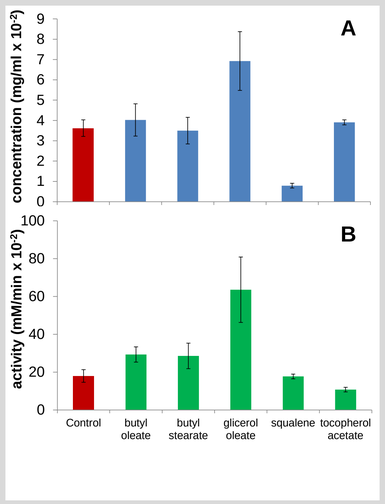


Figure 8. The effect of cuticular fatty acids on protein concentration **(A)** and *C. coronatus* elastase activity **(B)**

RESULTS (CONT.)



Figur 9. The effect of other substances detected in insect cuticle on protein concentration **(A)** and *C. coronatus* elastase activity **(B)**

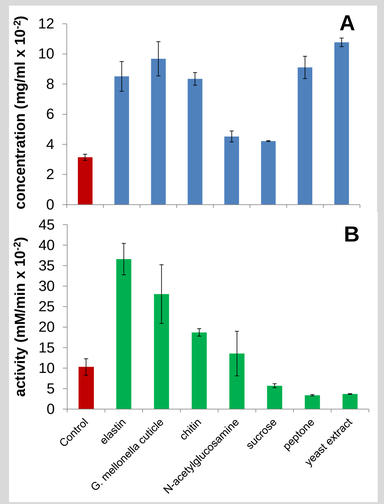


Figure 10. The impact of various C and/or N sources on protein concentration **(A)** and *C. coronatus* elastase activity **(B)**

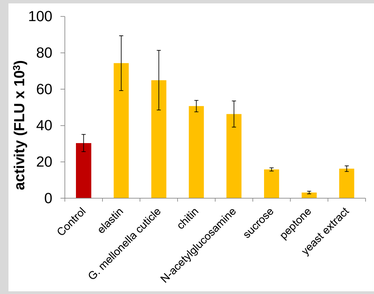


Figure 11. The effect of various C and/or N sources on *C. coronatus* proteolytic activity

Obtained results showed that addition of fatty alcohols C20, C28 and glycerol oleate stimulate *C. coronatus* to release into the culture medium high amounts of proteins. In contrast, fatty alcohols C10, C18, C20, C30 and squalene reduced the ability of entomopathogenic fungus to secretion of proteins **(Figures 7A, 9A)**. Low molecular weight fatty alcohols (C10-14) as well as alcohol C30 decreased the activity of elastase **(Figure 7B)**. **Figure 9B** shows that propagation of *C. coronatus* in media supplemented with butyl oleate, butyl stearate or glycerol oleate resulted in elevated elastase activity.

MM supplementation with C13:0, C19:0 and C20:4 fatty acids caused an increase in protein content, whereas addition of C22:0, C19:1 and C20:5 decreased it **(Figure 8A)**. In contrast to fatty alcohols, MM supplementation with fatty acids resulted in substantial increase of elastase activity, with the exception of C26:0 **(Figure 8B)**.

Addition of various C and/or N sources increased secretion of proteins. **Figure 10A** showed that the complex and rich in proteins additives i.e. yeast extract, cuticle from *G. mellonella* larvae, peptone, elastin and chitin caused significant increase in protein content. The highest elastase activity was observed in the medium supplemented with elastin (specific substrate for elastase). Addition of *G. mellonella* cuticle and chitin induced elastolytic activity of *C. coronatus*. In contrast, addition of peptone and yeast extract as well as sucrose (additives not present in the cuticle of insects) resulted in decreased elastase activity **(Figure 10B)**. Similar results were obtained for the measurements of total proteolytic activity **(Figure 11)** indicating the leading role of elastase. .

DISCUSSION

The increase in activity of elastase in the presence of most tested here cuticular fatty acids, suggests that during the infection process cuticular fatty acids may stimulate the fungus to overproduction of this enzyme. High proteolytic activity of entomopathogenic fungi is commonly considered as a key factor in the virulence. As a result of elevated fungus elastolytic activity a chance of successful mycosis increases. On the other hand, the presence in the cuticle of fatty alcohols C10-14 and C30, which decrease the activity of elastase, might protect insect from the fungus intrusion into hemocoel.

Significant decrease in amount of proteins released from mycelia and decrease of elastase activity in the presence of fatty alcohols C10-C14 suggests that these compounds might play a crucial role in protecting insect cuticle against penetration by the *C. coronatus*. In insect's resistance to fungal infection cuticular alcohols C24 and C30 as well as tocopherol acetate might be engaged. On the other hand, glycerol oleate, butyl oleate, and butyl stearate as well as most of tested here fatty acids can accelerate the development of pathogenesis.

Increase in elastase activity and protein content in the presence of elastin (one of main proteins found in the cuticle of many insect species) suggests that elastin might be responsible for the susceptibility of insects to infections, since it can be easily metabolized by the fungus.

Similar effect was observed with addition of *G. mellonella* cuticle (larvae are efficiently attacked by *C. cornatus*). Low elastase activity in the medium supplemented with rich in proteins additives (not found in the cuticle, but regulating elastase activity) may suggest that the fungus uses these substances to increase biomass and switch to saprotrophic mode of life. The decrease of elastase activity in the presence of sucrose, may indicate the occurrence of catabolic repression.

There is a need of more studies, which would explain molecular and cellular processes underlying susceptibility/resistance of insects to *C. coronatus* infection.

CONCLUSION

1. Cuticular fatty alcohols, fatty acids and other substances not belonging to the main groups of lipids have diverse effects on protein content and activity of *C. coronatus* elastase.
2. C and/or N sources (both, identified and not identified in the insect's cuticle) are regulators of *C. coronatus* elastase activity.
3. Susceptibility or resistance of various insect species to *C. coronatus* invasion depends on the species-specific cuticle composition.

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