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Antibacterial and antifungal lysozyme-type activity in *Cameraria ohridella* pupae

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Abstract

Lysozyme-type antibacterial and antifungal activity in pupae of *Cameraria ohridella* was studied. Activity against *Micrococcus luteus* and *Bacillus megaterium* was detected in pupae extract. Also antifungal activity from *C. ohridella* pupae extract directed against *Saccharomyces cerevisiae* strain W 303 was shown. During immunoblotting two bands in pupae extract, with molecular mass of about 15 and 28 kDa were recognized by antibodies directed against HEWL. After acid electrophoresis followed by bioautography of the extract, two lytic zones showing lysozyme-type activity against *M. luteus* were observed. Two bacteria: Gram-positive *Aerococcus viridans* and Gram-negative *Aeromonas salmonicida* ssp. *masoucida* were isolated from pupae of *C. ohridella*. Their activity against *M. luteus*, *B. megaterium*, and *S. cerevisiae* W303 was detected. After immunoblotting with antibodies against HEWL, also two proteins from bacterial suspensions of *A. viridans* and *A. salmonicida* were detected, about 15 and 28 kDa.

Keywords: *Cameraria ohridella*; Lysozyme; Antibacterial activity; Antifungal activity; *Aerococcus viridans*; *Aeromonas salmonicida* ssp. *masoucida*

1. Introduction

The first antibacterial factor purified from insect hemolymph was lysozyme (Powning and Davidson, 1976). It is an important antibacterial and antifungal protein in the insect immune mechanism. Lysozyme is a bacteriolytic enzyme that hydrolyzes β-1,4-glycosidic linkages between *N*-acetylglucosamine and *N*-acetylmuramic acid of the peptidoglycan layer in the bacterial cell wall. Lysozyme is a part of the defense system against bacteria and has been described in most animals (Dunn, 1986; Jollès and Jollès, 1984). Its presence in the hemolymph was first demonstrated in honeybees (Mohrig and Messner, 1968a) and then in other holometabolic and hemimetabolic insect species, e.g., in laboratory waxmoth *Galleria mellonella* (Powning and Davidson, 1976), in silkworm *Bombyx mori* (Abraham et al., 1995), in cricket *Gryllus bimaculatus* (Schneider, 1985), also in *Hyalophora cecropia* (Hultmark et al., 1980), *Manduca sexta* (Spies et al., 1986), *Heliothis virescens* (Lockey and Ourth, 1996) and in other insects. The lysozymes purified from hemolymph have molecular weights and properties similar to those of hen egg white lysozyme (HEWL), but show significantly higher enzymatic activity.

The enzyme was found in the guts of several insects: *Rhodnius prolixus* (Ribeiro and Pereira, 1984), *Musca domestica* (Ito et al., 1995; Lemos et al., 1993), *Drosophila melanogaster* (Kylsten et al., 1992), and in the salivary glands of mosquitoes *Anopheles darlingi* (Moreira-Ferro et al., 1998) and *Aedes aegypti* (Rossignol and Lueders, 1986). Lysozyme from eggs of the dipterous fly *Ceratitis capitata* was isolated and characterized (Fernandez-Sousa et al., 1977). The lysozyme activity was also detected in insect hemocytes (Anderson and Cook, 1979).

Besides its muramidase activity, lysozyme exerts fungistatic or fungicidal properties. Lysozyme hydrolyzes

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the β-1,4 linkages in chito-oligosaccharides in fungal cell wall. The influence of this enzyme on fungi was studied more widely. Lysozyme purified from the hemolymph of *G. mellonella* larvae inhibits the growth of yeasts in vitro; hen egg white lysozyme and human lysozyme also show this inhibiting effect (Vilcinskas and Matha, 1997).

Cameraria ohridella is the most dangerous horse-chestnut's (*Aesculus hippocastanum* L.) pest. This common leafminer occurs also on the maple-trees (*Acer pseudoplatanus* and *A. platanoides*). Larvae of this moth destroy parenchyma of leaves. They form hudge mines in which they pupate. Trees after heavily defoliation have a drastically shortened period of photosynthesis, what disturbs the process of accumulating enough reserve materials for winter. A few years of a such endanger can eventually cause the death of trees (Gregor et al., 1998; Skuhravy, 1999). Pupae are dark brown, 3.5–5.0 mm long and stay inside the leaves. The last generation of pupae spends the winter inside the fallen leaves.

Cameraria ohridella spread gradually from the Macedonia (Deschka and Dimic, 1984), to Austria, Hungary, Germany, Slovakia, Czech Republic, Italy, France, and Poland. Other parts of the continental Europe are predicted to be infested in a few more years. The large scale of the spread is caused by almost total absence of natural enemies, because it is important to control *C. ohridella* populations (Grabenweger and Lethmayer, 1999; Sefrova, 2003). Insecticides (Skuhravy, 1999) and pheromones can be used to control this pest (Svatoš et al., 2001). Nowadays, studying of *C. ohridella*'s immune defence is necessary.

In this study, we tested the effect of the extract from *C. ohridella* pupae against bacteria *M. luteus*, *B. megaterium* and on the growth of yeast *S. cerevisiae*. The lysozyme-type activity in pupae extract was shown. Additionally two bacteria species with similar lysozyme-like activity were isolated from *C. ohridella* pupae.

2. Materials and methods

2.1. Insects collection and extract preparation

Pupae of *C. ohridella* were collected from *A. hippocastanum L.* in the Lublin area (Poland), during August 2003 and 2004, with total number of about 2000 specimens. Pupae were isolated from fresh collected leaves and frozen in the liquid nitrogen, then washed in 70% ethanol. The whole insects (50 individuals) were homogenized as described (Guedes et al., 1997) and supernatant was used for enzyme assays. Pupae were homogenized in glass homogenizer, in an ice bath, with 0.01 M sodium phosphate buffer (pH 6.4), containing protease inhibitors: 1 mM PMSF and Complete-Protease Inhibitor Cocktail Tablets (Roche), dissolved as recommended and added 20 μl/1 ml. The homogenate was then centrifuged at 14000 rpm for 10 min at 4 °C. For antibacterial and antifungal assays, the supernatant was filtered through 0.22-μm pore size filter (Millipore) to remove any remaining microorganisms.

The extracts were obtained from three groups of *C. ohridella* pupae treated as followed: I—pupae were isolated from leaves and immediately frozen in liquid nitrogen, II—torn off leaves were kept for 24 h at room temperature and after that pupae were isolated and frozen, III—pupae were isolated from leaves and kept free for 24 h in Petri dishes at room temperature before freezing. All three extracts were used for estimation of lytic activity against *M. luteus*. In other experiments the extract from control insects (group I) was used.

2.2. Assays of antibacterial activity of C. ohridella pupae extract

The activity against Gram-positive bacteria *M. luteus* was determined in turbidimetric assay (Shugar, 1952) with freeze-dried cell walls of *M. luteus* (Sigma), using suspension of dead cells (1 mg/ml) in 33 mM phosphate buffer, pH 6.4. The rate of cell wall hydrolysis was measured after 15 s incubation at 25 °C at 450 nm in Bio-Rad spectrophotometer. The enzyme activity was calculated in units/mg. The concentration of protein was estimated using the Bradford reagent (Bio-Rad) (Bradford, 1976). Bovine serum albumin was used as a standard. A unit of activity was defined as the change in the absorbance of 0.001 per 10 min, under the above conditions. Lysozyme activity was expressed as U/mg.

Bacteria *B. megaterium* in exponential phase of growth in 5 μl of suspension (OD 1.0) was added to 500 μl broth containing the extract of pupae (protein concentrations: 0.17 and 0.68 mg/ml) or standard lysozyme—HEWL (0.40 mg/ml). Samples were incubated with shaking at 37 °C for 20 h. Then, the mixtures were rapidly chilled and A_{450} were measured.

Bactericidal activity was measured also by liquid broth method. *B. megaterium* in exponential phase of growth was added to broth with extract pupae or HEWL as above and samples were incubated at 37 °C for 1 h, and then different dilutions (in 0.85% saline) were spot inoculated on a nutrient agar. Colony forming units (CFU) were counted after incubation at 37 °C for 24 h.

2.3. Assay of antifungal activity of C. ohridella pupae extract

Saccharomyces cerevisiae strain W 303 was grown overnight at 30 °C in 10 ml of liquid YPG medium. After incubation the density of yeast cells was determined by measuring the extinction at A_{600}. For anti-yeast activity of pupae extracts, 10 μl suspension of yeast (OD 4.0), various concentration of extract and

5 μl water solution of streptomycin sulphate (50 μg/ml) were mixed in 500 μl medium (0.1% yeast extract, 0.2% glucose, and 0.05% peptone dissolved in phosphate buffer 0.01 M, pH 6.4). The changes of A_{600} were measured (Bio-Rad spectrophotometer) directly after mixing and after 20 h of incubation at 300 °C. The fungal cells were observed under Olympus Provis AX70 microscope (magnification 1000×).

2.4. Polyacrylamide gel electrophoresis

Analytical electrophoresis of proteins was carried out with an accordance to Reisfeld et al. (1962) in 15% separation gel at pH 4.3 for 1 h at 150 V. The lysozymes were visualized using an activity assay—bioautography. At first the gel was washed in 33 mM phosphate buffer, pH 6.4 for 30 min. This was followed by overlaying the gel with 10 ml phosphate buffer containing 10 mg freeze-dried cells of *M. luteus* in 1% agarose. The lysozyme bands were detected after 48 h by clearing of bacteria in the overlaid in agarose gel. Hen egg white lysozyme was used as a standard.

SDS–polyacrylamide gel electrophoresis was performed by the method of Laemmli (1970) in 12% polyacrylamide gels for 1.5 h at 120 V. Samples containing 25 μg of protein were heated at 100 °C for 10 min in sample buffer and used for electrophoresis. Hen egg white lysozyme was used as a standard.

Bacteria isolated from pupae were multiplicated in 3 ml of liquid Hawiger medium (Jeljaszewicz et al., 1978) and 20 μl of bacterial suspension with 5 μl sample buffer were used for electrophoresis. For determination of molecular mass of detected proteins, ladder (Fermentas) and HEWL (Sigma) were used.

2.5. Immunoblotting

After SDS–PAGE proteins were electroblotted onto Immobilon membrane (Millipore) for 90 min at 150 V. For identification of the lysozyme, polyclonal antibodies against HEWL in dilution 1:1000 were used. As second antibodies, alkaline phosphatase-conjugated goat anti-rabbit IgGs were used. Immunoreactive bands were visualized by incubation with *p*-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.

2.6. Bacterial isolation

To isolate microorganisms producing the lysozyme, the Hawiger plates containing (in 0.066 M phosphate Sörensen's buffer at pH 6.4): 1.5% dry meat broth; 0.4% yeast extract; 0.6% D-glucose; 0.1% Tween 80; 1.7% agar–agar, and 0.1% freeze-dried of *M. luteus* cells (Sigma) (heat-killed) were applied (Jeljaszewicz et al., 1978). The homogenate was prepared from 50 pupae in weight proportion of 1:4 (1 part of masses of pupae and 4 parts of phosphate buffer). The homogenate was centrifuged for 10 min at 6000 rpm. Then, the extract was inoculated (50 μl) on Hawiger plate and incubated at 30 °C for 72 h. The colonies surrounded by the lytic zones were chosen for bacteria identification.

2.7. Bacterial identification

For identification of bacteria, API 20 STREP identification system for *Streptococci* (bioMérieux) and API 20 NE identification system for non-fastidious, Gram-negative rods (bioMérieux)—standardized methods combining 20 biochemical tests were used.

2.8. Determination of bacterial lysozyme-like activity

Bacteria *Aerococcus viridans* and *Aeromonas salmonicida* ssp. *masoucida* were multiplicated in liquid Hawiger medium at 37 °C with shaking and cultures were centrifuged at 2200 rpm for 60 min. The activity of the lysozyme was determined in a supernatant of bacterial culture according to Hawiger (1968). Supernatant (3 ml) was filtered through 0.22-μm pore size filter (Millipore) and mixed with 3 ml of the standard suspension of *M. luteus* cells. Optical density A_{550} was measured immediately after mixing (control) and after 10 min incubation time at 37 °C. Cell wall suspension was prepared as follows: freeze-dried *Micrococcus* cells heat-killed (1 mg/10 ml) was dissolved in Sörensen's sodium phosphate buffer 0.066 M (pH 6.4). The concentration of protein in supernatant of bacteria culture was estimated using the Bradford reagent (Bio-Rad) (Bradford, 1976). Bovine serum albumin was used as a standard. One unit of activity (U) was defined as the change in absorbance of 0.001 per 10 min, under the above conditions. Lysozyme activity was expressed as U/mg.

Antimicrobial activity of *A. viridans* and *A. salmonicida* against *B. megaterium* and *S. cerevisiae* was detected by disc-diffusion method in Hawiger's medium.

2.9. Statistical analysis

Cohran–Cox test was used to determinate statistical significance. The differences between statistical parameters were considered significant when $p < 0.05$.

3. Results

3.1. Antibacterial and antifungal activity of C. ohridella pupae extract

Generally, bacteriolytic action of lysozyme is particularly strong against strains of *Micrococcus*, *Bacillus*, and *Sarcina*. Therefore, *M. luteus* and *B. megaterium* as very

sensitive strains for lysozyme action were used. In our research, the lysozyme activity against *M. luteus* in extract of *C. ohridella* pupae was estimated. The studies were performed on three groups of individuals. In group I, immediately isolated from leaves and frozen in liquid nitrogen (Table 1) lysozyme activity amounted 90.2 U/mg. In extract of pupae from group II—pupae kept in torn off leaves for 24 h at room temperature before freezing, lysozyme activity increased to 182.5 U/mg, and in the third group of insects (III)—pupae isolated from leaves and kept free for 24 h in Petri dishes at room temperature before freezing, activity of lysozyme amounted 177.1 U/mg. Both, taking pupae from leaves and incubating them in leaves increased about twice lysozyme-like activity in extract of pupae.

Activity of *C. ohridella* extract against *B. megaterium* was detected. The addition of the extract in protein concentration 0.17 mg/ml inhibited *B. megaterium* growth in 22%. At higher protein concentration (0.68 mg/ml) bacterial growth was inhibited in 53% (Fig. 1). Lysozyme (HEWL) 0.40 mg/ml inhibited multiplication of *B. megaterium* in 47%. The results indicated inhibition of *B. megaterium* by the extract were confirmed using liquid broth method (Table. 2).

In our studies, the antifungal activity of the extract from *C. ohridella* pupae against *S. cerevisiae* W 303 was detected. The extract repressed the fungal proliferation measured by turbidimetric assay (Fig. 2). The addition of the extract in protein concentration 0.17 mg/ml inhibited the fungal cell proliferation by 52%. At higher concentration inhibition reached at 0.34 mg/ml—67% and at 0.52 mg/ml—81%. Standard lysozyme (0.10 mg/ml) inhibited *S. cerevisiae* growth by 71% and 0.20 mg/ml almost completely (95%). In Fig. 3 was showed formation of yeast's cell-clusters in the presence of the higher concentration of the proteins from extract as well as in the presence of hen egg white lysozyme, used as the control. During the exposition to the extract, cells of *S. cerevisiae* W 303 became ballooned and deformed.

When native proteins of pupae extract were analyzed by electrophoresis at pH 4.3 and subsequent bioautography (Fig. 4), two lytic zones indicating lysozyme-type

Table 1
The bacteriolytic lysozyme activity in extract of *C. ohridella* pupae

Lysozyme activity in extract of C. ohridella pupae, Ulmg (±*SD*)	
Control group of pupae	90.2 (±1.27)
Pupae incubated in leaves at room temperature for 24 h	182.5 (±1.58)*
Pupae taken out from leaves and incubated at room temperature for 24 h	177.1 (±1.16)*

Samples were incubated for 15 s in 33 mM phosphate buffer and then assayed turbidimetrically at 25 °C for bacteriolytic activity against cell walls of *M. luteus*.

* Values were significantly different from the control value ($p < 0.05$, Cohran–Cox test).

Table 2
Inhibition of *B. megaterium* growth after incubation for 1 h at 37 °C with extract from *C. ohridella* pupae or with HEWL

Treatment	Bacterial counts CFU/ml (±SD)
Bacillus megaterium (control)	1.5×10^5 (±0.36)
Bacillus megaterium with *C. ohridella* extract (0.68 mg/ml)	8.6×10^3 (±1.37)*
Bacillus megaterium with HEWL (0.40 mg/ml)	4.4×10^3 (±1.59)*

* Values were significantly different from the control value ($p < 0.05$, Cohran–Cox test).

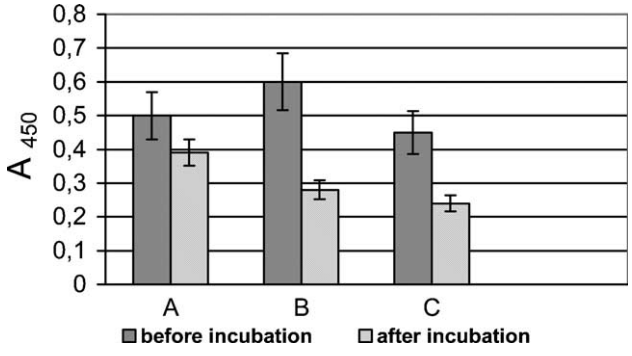

Fig. 1. Inhibition of *B. megaterium* growth after incubation for 20 h at 37 °C with extract from *C. ohridella* pupae or with HEWL. Bars represent ±SD calculated from five independent experiments. (A) *B. megaterium* with 0.17 mg/ml extract, (B) *B. megaterium* with 0.68 mg/ml extract, and (C) *B. megaterium* with 0.40 mg/ml HEWL.

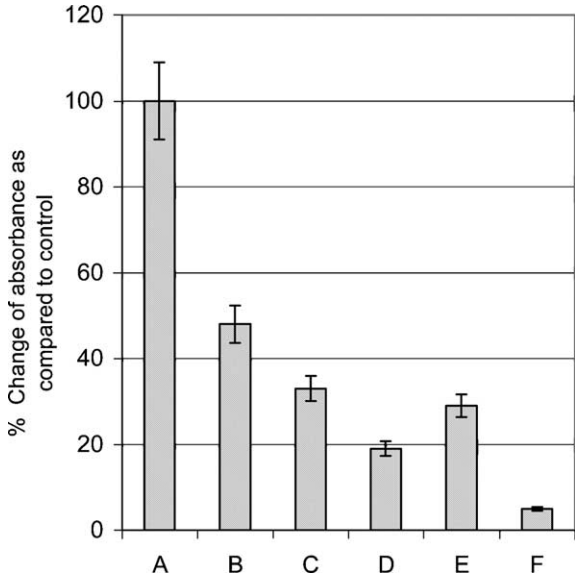

Fig. 2. Inhibition of *S. cerevisiae* growth expressed in % change of absorbance A_{600} after incubation for 20 h at 30 °C with increasing amounts of extract from *C. ohridella* pupae or with HEWL. Bars represent ±SD calculated from five independent experiments. (A) *S. cerevisiae*-control group without extract, (B) *S. cerevisiae* with 0.17 mg/ml extract, (C) *S. cerevisiae* with 0.34 mg/ml extract, (D) *S. cerevisiae* with 0.52 mg/ml extract, (E) *S. cerevisiae* with 0.10 mg/ml HEWL, and (F) *S. cerevisiae* with 0.20 mg/ml HEWL.

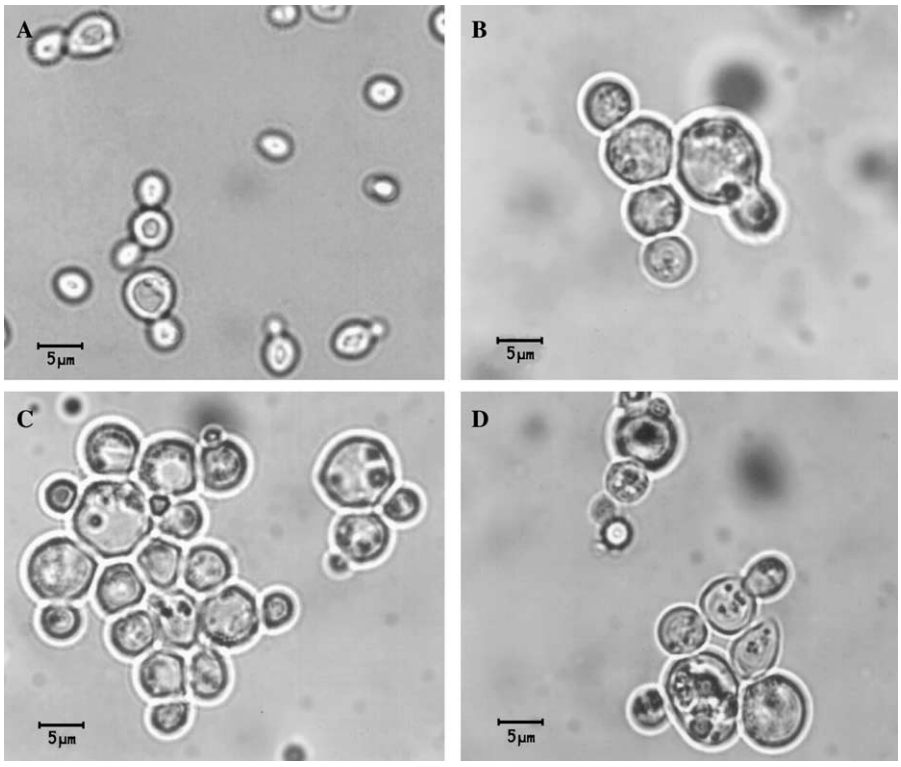

Fig. 3. *Saccharomyces cerevisiae* cells (magnification 1000×) observed after incubation for 20 h at 30 °C, with increasing amounts of extract from *C. ohridella* pupae or with HEWL. (A) *S. cerevisiae*-control group without extract, (B) *S. cerevisiae* with 1.03 mg/ml extract, (C) *S. cerevisiae* with 1.72 mg/ml extract, and (D) *S. cerevisiae* with 0.20 mg/ml HEWL.

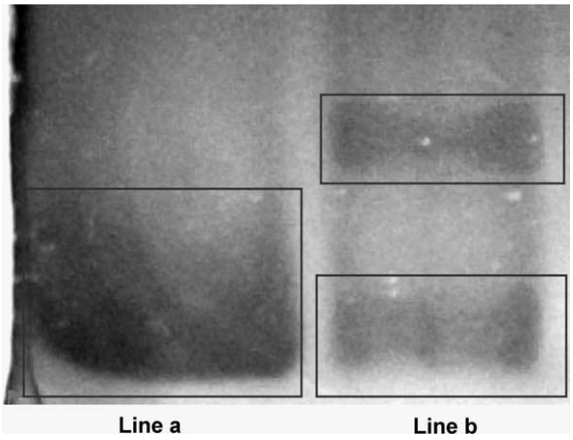

Fig. 4. Analytical electrophoresis at pH 4.3 with bioautography shows bacteriolytic activity of extract from *C. ohridella* pupae. The basic proteins migrate towards the cathode (bottom), the acid protein was localized higher than basic proteins. Line a—HEWL; b—extract from *C. ohridella* pupae. Rectangles indicate zones of less density caused by bacteriolytic activity against *M. luteus*.

activity against *M. luteus* were observed. One of them migrated towards the cathode like HEWL, whilst the second one migrated slower. Sample of HEWL created only a single lytic zone and migrated towards the cathode to the bottom of the gel as a basic protein. The second zone localized higher than HEWL zone could originate from another form of lysozyme from *C. ohridella* pupae.

We found that antibodies directed against hen egg white lysozyme during immunoblotting recognized two proteins from the extract. Their molecular masses were estimated at about 15 and 28 kDa (Fig. 5).

3.2. Antibacterial and antifungal activity of bacteria isolated from C. ohridella pupae

The presence of microorganisms in the *C. ohridella* pupae caused, that we decided to perform isolation and identification of bacteria from *C. ohridella* pupae extract and from the gut of this insect. In our samples, we identified two species of bacteria, secreting lysozyme-like substance: *A. viridans* and *Aeromonas salmonicida* ssp. *masoucida–achromogenes*.

Aerococcus viridans indicated activity against *M. luteus* on Hawiger plates. Lytic effect against *M. luteus* was shown in Fig. 6. The lysozyme-type activity against *M. luteus* was also caused by *A. salmonicida* ssp. *masoucida* detected in the extract of *C. ohridella* pupae. Bacteria *A. salmonicida* was isolated on Hawiger medium and identified using API 20 NE. Lytic effect against *M. luteus* on Hawiger plate caused by *A. salmonicida* (Fig. 7) was smaller then bacteriolitic effect of *A. viridans*. The lysozyme-type lytic activity of both bacteria against

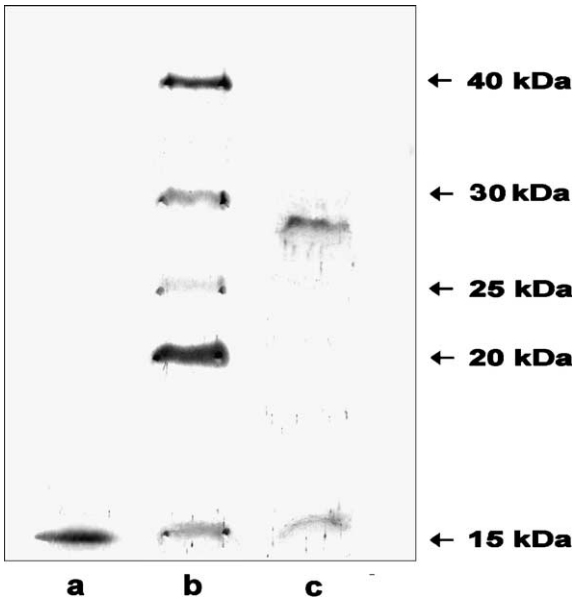

Fig. 5. Immunoblotting with monoclonal antibodies against hen egg white lysozyme (HEWL). Line a—HEWL, b—ladder, and c—extract of *C. ohridella* pupae.

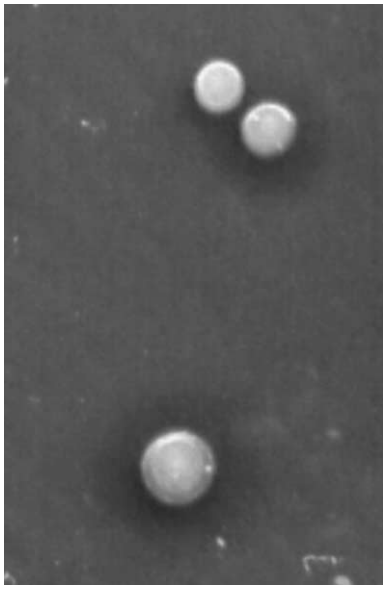

Fig. 6. Bacteriolytic activity against *M. luteus* caused by growing *A. viridans*; lytic effect of bacterial colonies on Hawiger plate.

M. luteus was confirmed by turbidimetric assay. The activity of *A. viridans* amounted 53.75 U/mg (SD ± 2.43) and of *A. salmonicida* was determined to 32.86 U/mg (SD ±2.82). ±SD calculated from four independent experiments.

The supernatant of *A. viridans* culture inhibited growth of *B. megaterium* (Fig. 8) and *S. cerevisiae* W 303 (Fig. 9). Supernatant of *A. salmonicida* shows similar inhibition effects.

After immunoblotting with antibodies against HEWL, two proteins from bacterial suspensions of

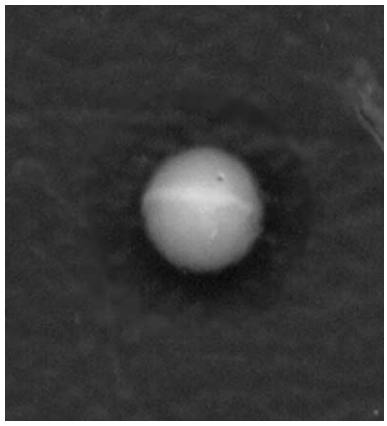

Fig. 7. Lytic zone against *M. luteus* caused by *A. salmonicida* on Hawiger plate.

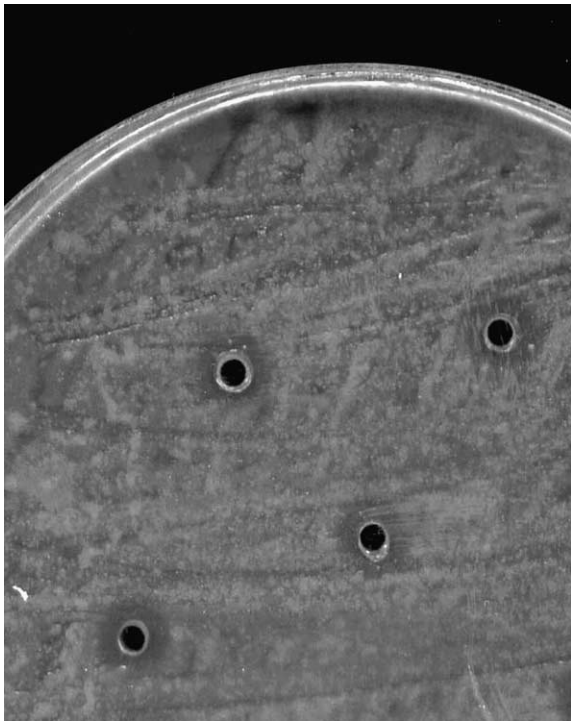

Fig. 8. Inhibition zones of *B. megaterium* growth caused by supernatant from *A. viridans* culture.

A. viridans and *A. salmonicida* were detected. The molecular mass of one of them was about 15 kDa, and about 28 kDa of the other (Fig. 10).

4. Discussion

It is known that lysozyme is a major factor in insects immune response (Mohrig and Messner, 1968b). The lysozyme could originate from various tissues of insect. In native insect *M. sexta*, the fat body contained the highest level of lysozyme RNA and lower in the other

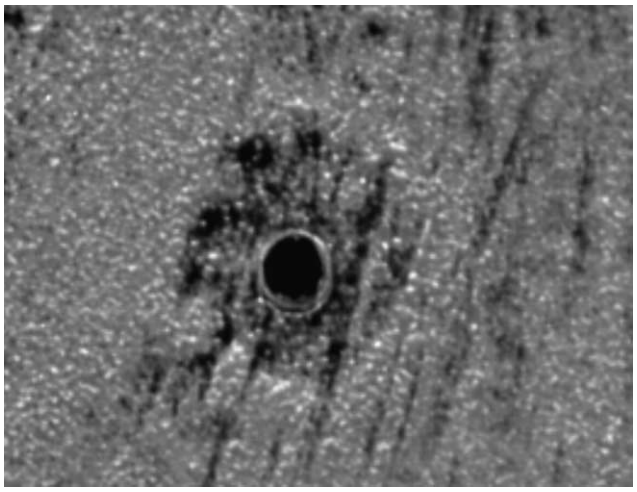

Fig. 9. Inhibition zones of *S. cerevisiae* growth caused by supernatant from *A. viridans* culture.

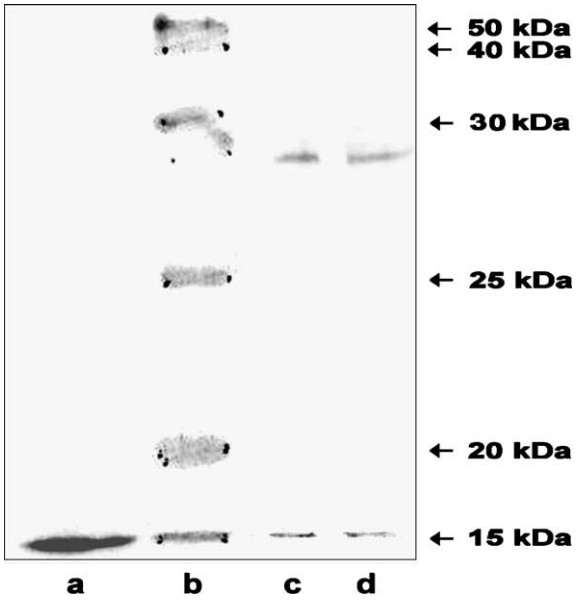

Fig. 10. Immunoblotting with monoclonal antibodies against hen egg white lysozyme (HEWL). Line a—HEWL, b—ladder, c—suspension of *A. viridans*, and d—suspension of *A. salmonicida*.

six tissues (midgut, heart pericardial cells, Malpighian tubules, epidermal tissues, salivary gland, muscle) (Mulnix and Dunn, 1993). The increase of lysozyme transcripts in seven tissues following treatment with peptidoglycan were detected in *M. sexta* and highest level of accumulation was observed in the fat body.

Other stimulators, including mechanical stress, increased significantly lysozyme-like activity in insects. Pupae of both lepidopteran species *G. mellonella* and *C. ohridella* were both sensitive to mechanical stress. Jarosz and Śpiewak (1979) analyzed changes of lysozyme activity after mechanical stress in *G. mellonella* pupae. The pupae were subject of several mechanical stimuli, such as: puncture, shelling, inversion and shelling plus inversion, and after all types of mechanical stimuli the increase of lysozyme level was observed. Therefore, in our researches similar immunizing effect in *C. ohridella* pupae, in II and III experimental groups, after a mechanical stimulation was observed. The pupae from I group also were exposed to slight mechanical treatment during isolation from leaves, probably insignificantly increasing the level of the lysozyme activity. However, the activities observed in pupae from group II and III were almost doubled comparing with initial level in group I.

Lysozyme-like activity in insects could originate from symbiotic or other microorganisms. The microorganisms in insects are located in various organs of their body: in the midgut, in the mycetomes in haemocoel or connected to midgut, in mycetocytes in fat body or in haemocoel, in Malpighian tubules, in ovaries, and eggs (Douglas, 1989). Bacteria *A. viridans* isolated from *C. ohridella* pupae gut and from the extract was also isolated and identified from the guts of Mexican fruitflies *Anastrepha ludens* (*Diptera*: *Tephritidae*) (Kuzina et al., 2001). The intestinal bacteria *Streptococcus faecalis* with similar lysozyme-like activity was detected in pupae of *G. mellonella* (Jarosz, 1975). Both *A. viridans* and *S. faecalis* are Gram-positive bacteria belonging to *Streptococci.*

The second one, isolated from gut, bacteria of *C. ohridella* was *A. salmonicida* spp. *masoucida*, Gram-negative. Sancho et al. (1996) isolated *A. salmonicida* as microflora associated to the larvae of human bot-fly *Dermatobia hominis* L. Jr. (*Diptera*: *Cuterebridae*). The Gram-negative rods were also found in *Diptera* and *Hymenoptera* midgut as mycetome symbionts (Douglas, 1989).

Both, the extract of *C. ohridella* pupae and supernatant of bacterial culture of *A. viridans* and *A. salmonicida* showed antibacterial activity against *M. luteus* and *B. megaterium.* HEWL used as a standard caused similar effect.

Insect lysozymes generally belong to the c-type, alike chicken egg white lysozyme (Yu et al., 2002), but sometimes to i-type (invertebrate type) lysozyme. Noteworthy is that eight lysozymes of *D. melanogaster* belong to c-type and three lysozymes belong to i-type lysozyme family (Bachali et al., 2002). In some insects, molecular mass of lysozymes differs from HEWL (14.4 kDa.) In *Locusta migratoria* (Zachary and Hoffmann, 1984) and *B. mori* (Powning and Davidson, 1973) molecular weight of lysozymes was determined to 16.5 kDa, but in *C. capitata* eggs to 23.2 kDa (Fernandez-Sousa et al., 1977). Three lysozymes from hemolymph of the cricket, *G. bimaculatus* were purified and their molecular weights to 11.1, 15.0, and 15.4 kDa were determined; after electrophoretic separation at pH 4.3 followed by bioautography, clear lytic activity for each enzyme was

observed (Schneider, 1985). It is possible that two different lysozymes are produced by gut bacteria of *C. ohridella* pupae. Analysis of *C. ohridella* extract using acid electrophoresis, indicated that two lytic zones and after immunoblotting of the extract and bacterial solution of *A. viridans* and *A. salmonicida*, also two proteins with mass about 15 and 28 kDa were recognized by antibodies against HEWL. Similarly, in other invertebrate, e.g. in oyster, after purification of plasma lysozyme and analyzing using SDS–PAGE, two bands with molecular weights of 18.2 and 38.8 kDa were identified (Chu and Van Veld, 2003).

We observed the antifungal activity of both, the extract from *C. ohridella* pupae and of the supernatant of the bacteria, showing lysozyme activity against *S. cerevisiae* W 303. Similar effect was observed by other authors. Wu et al. (1999) reported that yeast cells of *Candida albicans* exposed to lysozyme were ballooned, sometimes had invaginations and some cells appeared colapsed and deflated. The occurring of the invaginations, frequently among bud scars, indicate that cell wall components may be possible targets for lysozyme. The lysozyme induced similar morphological changes observed among other strains (Wu et al., 1999). The antifungal action of lysozyme in vitro against *C. albicans* was investigated also by Nishiyama et al. (2001). They reported that lysozyme inhibited separation of mature cells and cells with cell wall materials accumulated in periplasmic space were frequently observed by them.

On the basis of the obtained results we presume that lysozyme-like activity from gut bacteria of *C. ohridella* pupae could be a part of the insect defense mechanism against ingested bacteria and fungi. In the guts of insects, the proteolytic enzymes occurring in the intestinal fluid, increase the liberation of symbiotic bacteria lysozyme—similar to insect lysozyme (Jarosz, 1979). Ursic Bedoya et al. (2005) suggested that lysozymes play a dual role. The first one being the digestive manner to break down ingested bacteria in the gut, the second—as a defence response against pathogens that enter the haemocoel. They proposed defensive and digestive roles for lysozymes in *Aedes aegypti*. The role of lysozymes of *C. ohridella* could be similar, but since it has not been recognized yet, therefore, the lysozyme-like proteins of this species require further studies.

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