The chemical composition of egg plugs deposited by *Sitophilus granarius* L. females on grain

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Abstract

Over 20,000 egg plugs collected from infested wheat grain were subjected to chemical analysis. Elemental analysis showed a relatively high content of nitrogen (about 9%). It suggested that the predominant constituent of egg plugs is a protein. Spectrum obtained in ESI-MS analysis showed a series of peaks characteristic corresponding to protein of molecular weight 30073 Da. The appearance of other peaks in this spectrum suggests that studied protein is not homogenous. A sample of egg plugs incubated with pepsin yielded a complex mixture of peptides. The most abundant peak in the ESI-MS spectrum of enzymatic hydrolysis products corresponds to peptide Mw 4560.76 Da. Chemical analysis indicated that the main component of egg plugs is protein.

Keywords: egg plugs, chemical composition, *Sitophilus granarius*

1. Introduction

Egg deposition by insect females is considered a complicated behavioural process, depending on numerous factors. Selection of place for egg deposition definitely affects the population development rate and survival of a given species in the biocenosis. On the basis of chemical stimuli perceived by receptors of smell, taste and contact chemoreception senses, each female has to find a food source for newly hatched larvae and simultaneously to estimate its abundance and nutritional value. The following step of the behaviour sequence is cognition of whether another female from the same species deposited eggs earlier in the same product. For this purpose, marking pheromones, epideictic pheromones and kairomones left on eggs are used by females. Aquatic insects usually produce a gelatinous secretion covering deposited eggs. In cases of depositing eggs inside a product, females can perceive sounds sent by feeding larvae (Nufio and Papaj, 2001).

Substances covering individual insect eggs or egg clusters are produced by accessory reproductive glands (*Glandulae accessoriae*), also called sebaceous glands (*Glandulae sebaceae*). These glands are twin or non-twin structures, symmetrical or asymmetrical, and their ends are placed in a common oviduct (*Oviductus*). Their secretions contain: poisons, pheromones, special glue substances for attaching eggs to the substrate, and substances for covering the eggs (Chapman, 1971; Gillot, 2002, 2005). In the case of cockroaches (Blatodea) and praying mantides (Mantodea) gland secretions are used for cocoon formation, while tsetse flies (*Glossinia morsitans*) use them for larvae' feeding.

Granary weevil (*Sitophilus granarius* L.) females deposit eggs inside cereal grains (wheat, barley and oat) performing the following routines:

1. boring a hole in grains with their snouts,
2. inserting an ovipositor into the hole and placing one egg in it,
3. closing a grain hole with gelatinous hardening substance (egg-plugs).

Egg deposition process was best described in older German literature (Müller, 1928; Pavlakos, 1931; Kllnike, 1936; Andersen, 1938). Colleteral glands' structure of granary weevil is well known (Krautwig, 1930; Khan and Musgrave, 1969; Müller, 1928). However, chemical composition of gland secretions is only partially recognized (Cox et aI., 2000; Niewiada et aI., 2005). Kunike (1928) provided the first data on egg-plug chemistry: it is not soluble in water, alcohol or acetone, but is slightly soluble in alkalies and reacts in Molish reaction. Egg-plug are stained cherry red with fuchsin (Frankenfeld, 1948), purple with gentian violet (Goossens, 1949), and greenish yellow with barberine sulphate (Milner et al., 1950).

Egg-plugs may provide protection of to deposited eggs from predators and parasitoids, mechanical stimuli, and drying. It would be useful to determine whether they can be perceived by insect contact chemoreceptors and/or whether they contain volatile epideictic pheromones which influence oviposition behavior. Grain weevil females usually deposit one egg inside a cereal kernel, however, under certain conditions numerous egg-plugs are observed on the surface of grain without deposited eggs under them.

Volatile pheromones marking egg depositions have already been described for numerous insect species. Their main role is to warn that a given product has been already occupied and it is an insufficient resource for further oviposition. These compounds are called epideictic pheromones or pheromones hampering egg deposition. The evidence of marking pheromones was revealed for the following Curculionidae species: boll weevil (*Anthonomus grandis* Boheman.) (Hedin et aI., 1974), pine weevil (*Hylobius abietis* L.) (Nordlander et aI., 1977; Borg-Karlson et aI., 2006), cabbage seed weevil (*Ceutorrynchus assimilis* (Paykull.)) (Kozłowski et aI.,1983), and cherry weevil (*Furcipes rectirostris* L) (Kozłowski and Borkowski, 1990).

This paper reports the first chemical analysis of egg plugs collected from infested wheat grain. We believe that a knowledge of their chemical composition may be useful for further studies of the biological activity of the egg plugs in oviposition process.

2. Material and methods

2.1. Biological material

Substance covering deposited eggs was collected from grain with the use of preparation needle. Ten mg of the product (over 20,000 plugs) was subjected to chemical analysis.

2.2. Chemical analysis

The elemental analysis of the sample of egg plugs was performed at Laboratory of Elemental Analysis and Environmental Studies (University of Wroclaw). The mass spectrometric analysis was performed using an Apex-Qe 7T instrument (Bruker Daltonics, Germany) equipped with dual electrospray ionization (ESI) source. Spectra were recorded using aqueous solutions of acetonitrile (50%) and formic acid (1%). The potential between the spray needle and the orifice was set to 4.5 kV. In MS/MS mode, the quadruple was used to select the precursor ions, which were fragmented in the hexapole collision cell applying argon as a collision gas. The product ions were subsequently analyzed by the ICR mass analyzer. For CID MS/MS measurements, the voltage 20 V over the hexapole collision cell was applied. The spectra were analysed using Data Analysis™ and Biotools™ software (Bruker). Two mg of egg plugs were subjected to elemental analysis. The sample was not purified or fractionated before analysis. The analysis revealed relatively high content of nitrogen (9 ± 1%). Sample was not soluble in methanol. The egg plugs were sonicated in formic acid and then diluted with water formed a cloudy solution.

After filtration this solution was separated into three parts. Part 1 was treated with pepsin. The solution resulting from the enzymatic hydrolysis was analyzed by ESI-MS. Part 2 was diluted with acetonitrile (1:1) and directly analyzed by ESI-MS. Finally, Part 3 was freeze-dried. The product obtained by lyophilization has the appearance of white powder. The samples of hydrolyzed and unhydrolyzed extract were subjected to biological activity tests.

2.3. Biological activity tests

The lyophilized products (hydrolyzed and unhydrolyzed extracts) were dissolved in water and deposited on wheat kernels at the amount of 1 µL. Two experiments were carried out simultaneously: a choice test (KE) (l0 treated grains with the extract and 10 grains treated with the same amount of water in one Petri dish) and no-choice test (EE) (20 grains treated with extract in one Petri dish). The control treatment was 20 wheat kernels treated with water in one Petri dish (KK). Twenty granary weevils (l0 females and 10 males, 1 - 5 days old) were placed on previously prepared grains. After 10 days of feeding the number of egg-plugs was recorded on all experimental kernels. Each experiment was carried out in 5 replications.

3. Results and discussion

3.1. Chemical analysis

The average content of nitrogen in purified, dry protein is about 16%, but real, air dried samples (Chibnall, 1948) of various proteins contained 8.1 (amandin) to 13.3% (egg albumin, native). The decreased content of nitrogen in these samples is a result of water content and mineral compounds. The relatively high content of nitrogen in our sample suggests, that the main component of egg plugs is a protein.

The result of ESI-MS measurement of soluble egg plug fraction presented in Fig. 1 demonstrated very characteristic pattern consisting of a series of multiply charged positive ions (the charge varies from +20 to +36). The spectrum was deconvoluted by maximum entropy algorithm (Fig. 2), revealing a predominant component with molecular mass of 30,073 ± 5. The ESI-MS spectrum confirmed that protein is the main component of studied material.

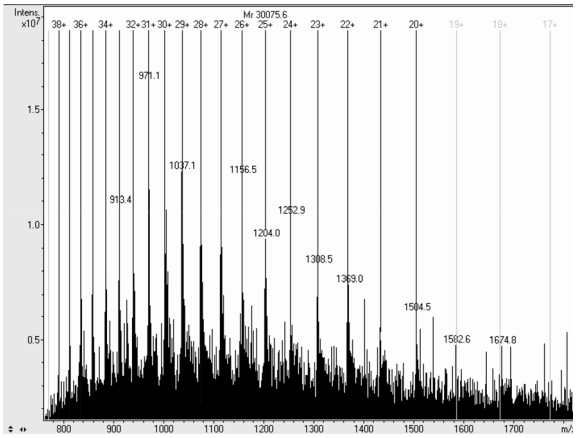

Figure 1 The ESI-MS spectrum of the protein extracted from egg plugs, with the peaks labeled with charges and m/z (mass to charge ratio).

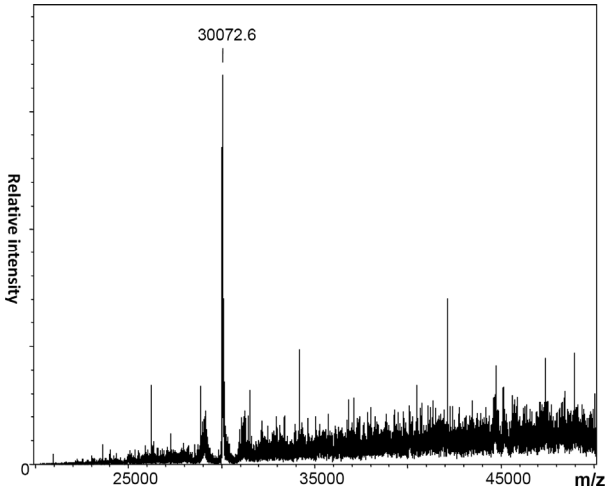

Figure 2 The deconvoluted ESI-MS spectrum of the protein extracted from eggs plugs.

The sample incubated with pepsin demonstrated a completely different spectrum character. Instead of a series of peaks with the charge ranging from +36 to +20, a series of relatively short peptides (the highest charge +7) was observed. The spectrum showed in Fig. 3 presented numerous fragments presumably formed by enzymatic hydrolysis of extracted protein. The susceptibility to the pepsin catalyzed hydrolysis is an another piece of evidence confirming proteic character of main egg plugs component.

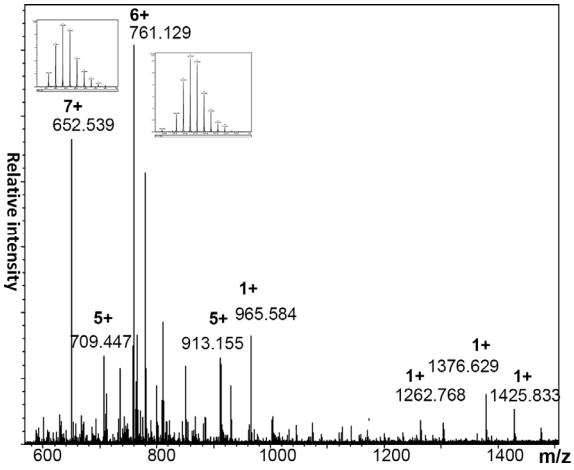

Figure 3 The ESI-MS spectrum of products obtained by pepsin catalyzed hydrolysis of egg plugs protein, with the isotopic distribution for two peaks of the highest abundances shown as inserts.

Selected peptide (m/z = 761.127) was subjected for MS/MS experiment. The fragmentation was performed by two methods: CID (collision induced dissociation) and ECD (electron capture dissociation). The obtained, CID fragmentation spectrum is presented in Fig. 4. The fragmentation pattern was interpreted using Biotools program (Bruker Daltonics, Germany). Although the sequence coverage was not sufficient to determine the whole sequence of the peptide, the program found many fragments , including: ANVVR; VGVV, VAPS. The analysis of fragmentation of peptide at m/z 761.127 suggested a high content of Val in investigated peptide.

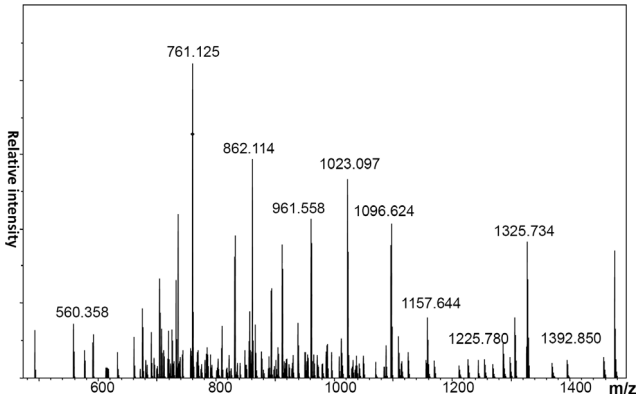

Figure 4 The ESI-MS/MS spectrum of most abundant component of peptic hydrolysis of egg plug protein.

3.2. Biological activity test

Results on biological activity of plug extracts are presented in Table 1. The numbers of egg plugs deposited on treated kernels was similar to that in control. Probably the applied amount of substance was not sufficient to be detected by S. granarius females. Further experiments should be performed with peptide derivatives synthesized on the basis the partial sequences of studied proteins.

Table 1 Number of egg plugs deposited by one female during 10 days on wheat kernels treated with hydrolyzed and unhydrolyzed extracts of plugs (SD in parenthesis)

	Number of egg plugs			
	Choice test		**No-choice test**	
	K	**E**	**KK**	**EE**
Hydrolyzed extract	15.5 (±2.1)	16.6 (±2.4)	15.0(±1.8)	14.7 (±1.3)
Unhydrolyzed extract	13.1 (±2.3)	10.8 (±2.8)	13.7 (±3.1)	11.6 (±2.0)

4. Conclusions

The chemical analysis revealed that the basic component of egg plugs produced by *S. granarius* is a protein with molecular mass 30,075. The mass spectrometry allowed us to find the partial sequences of the isolated protein.

The biological activity of egg plugs in oviposition process will be studied on the basis of knowledge their chemical composition.

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