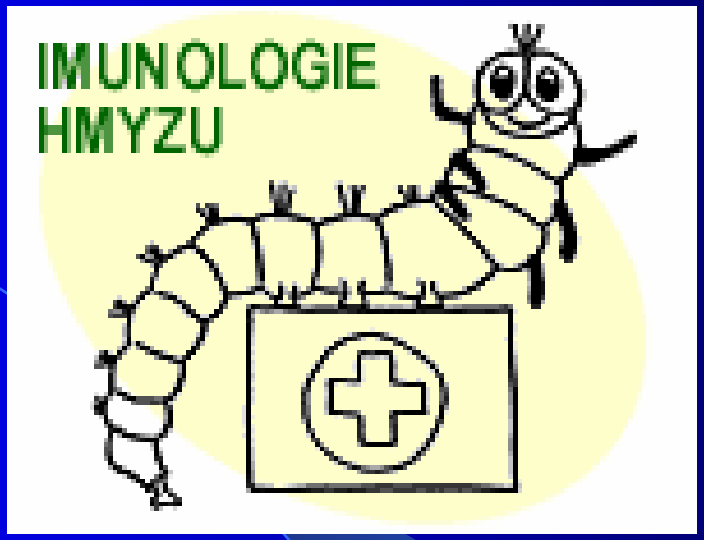
Laboratoř imunologie hmyzu



Pavel Hyršl

Oddělení fyziologie a imunologie živočichů

Ústav experimentální biologie

Přírodovědecká fakulta

Masarykova Univerzita v Brně

http://www.sci.muni.cz/ksfz/imuno_hmyz.html

IMUNITNÍ SYSTÉM HMYZU

BUNĚČNÁ IMUNITA ⟷ HUMORÁLNÍ IMUNITA

-pohyblivé krevní buňky – hemocyty

(počet a aktivita hemocytů je modulována humorálními faktory a neuroendokrinním systémem)

- koagulace
- fagocytóza (oxidativní vzplanutí?)
- nodulace
- enkapsulace

- molekuly v hemolymfě
- antigenem indukované antibakteriální a regulační faktory

- aglutininy
- koagulační kaskáda
- fenoloxidásová kaskáda
- baktericidní peptidy
- antimikrobiální faktory (lektiny, hemolin)
- lysozym

stresové faktory

Ukázka klasifikačních schemat hmyzích hemocytů

Classification scheme	Prohaemocytes	Plasmatocytes	Authors equivalent classes to: Granular cells	Cystocytes	Spherule cells	Oenocytoids
Hollande (1911)	proleucocytes	phagocytes	granular leucocytes adipoleucocytes		spherule cells	oenocytoids
Metalnikov (1924)	lymphocytes proleucocytes	← leucocytes →			cells spheruleuses	
Cameron (1934)	lymphoocytes	leucocytes	leucocytes?		spherule cells	oenocytes
Yeager (1945)	proleucocytoids	plasmatocytes podocytes vermiform cells	chromophilic cells?	cystocytes	spheroidocytes	oenocyte-like cells
Wigglesworth (1959)	proleucocytes	phagocytic amoebocytes			phagocytic amoebocytes?	oenocytoids
Jones (1962) and Arnold (1974)	prohemocytes	plasmatocytes podocytes vermiform cells	granular hemocytes adipohemocytes	cystocytes	spherule cells	oenocytoids
Brehélin et al. (1978)	prohemocytes	plasmatocytes granulocytes? thrombocytoids podocytes	granulocytes	coagulocytes	spherule cells	oenocytoids
Gupta (1979)	prohemocytes	plasmatocytes	granulocytes	coagulocytes	spherulocytes	oenocytoids

Svobodová 1995

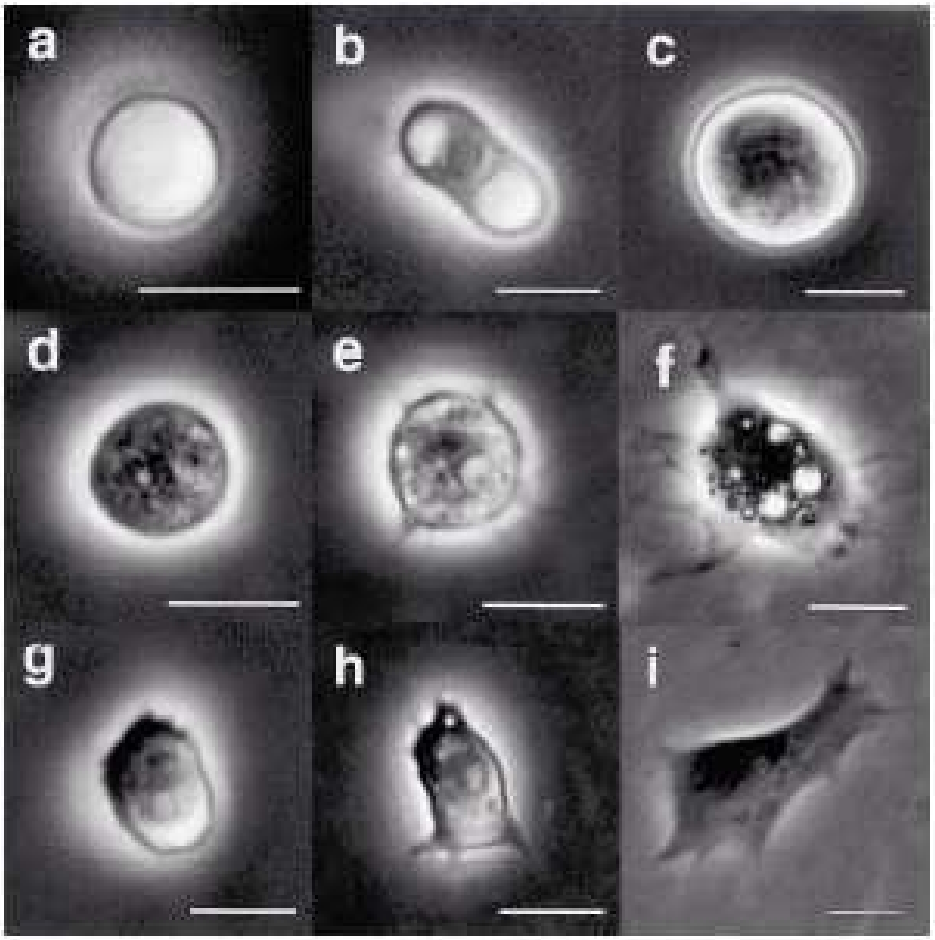


Fig. 1. Morphology of different types of hemocytes first placed into culture. (a) Prohemocyte, (b) spherulocyte, (c) oenocytoid, unlysed, (d) granulocyte, after 10 min of culture, (e) granulocyte, which partially protruded filopodia, after 20 min of culture, (f) fully spread granulocyte, after 40 min of culture, (g) plasmatocyte, after 10 min of culture, (h) early-spreading plasmatocyte, after 2 h of culture, and (i) fully spread plasmatocyte, after 3 h of culture. Scale bar=10 μm.

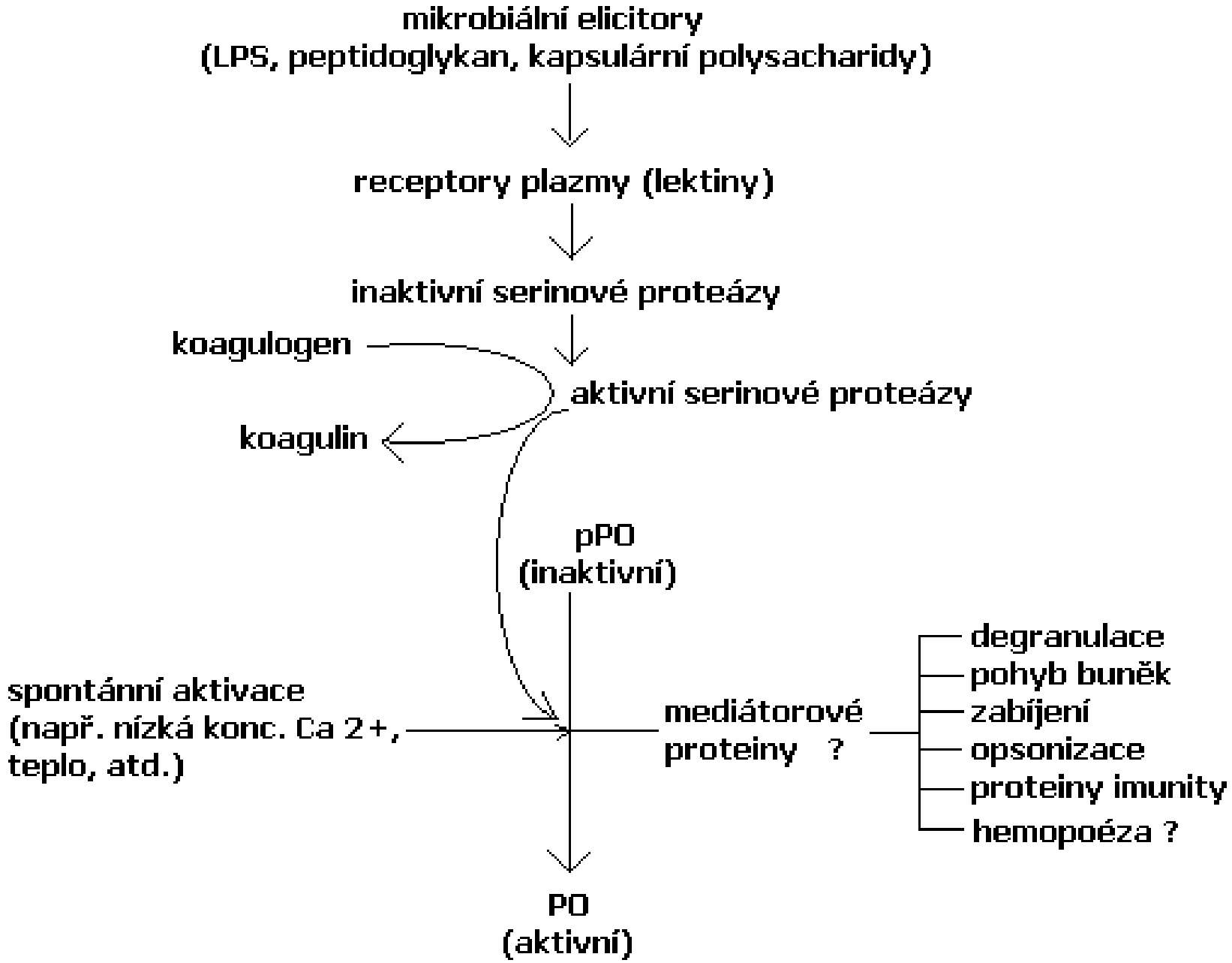
Hemocyty *B. mori* podle Yamashita & Iwabuchi, 2001.

Hmyzí hemocyty:

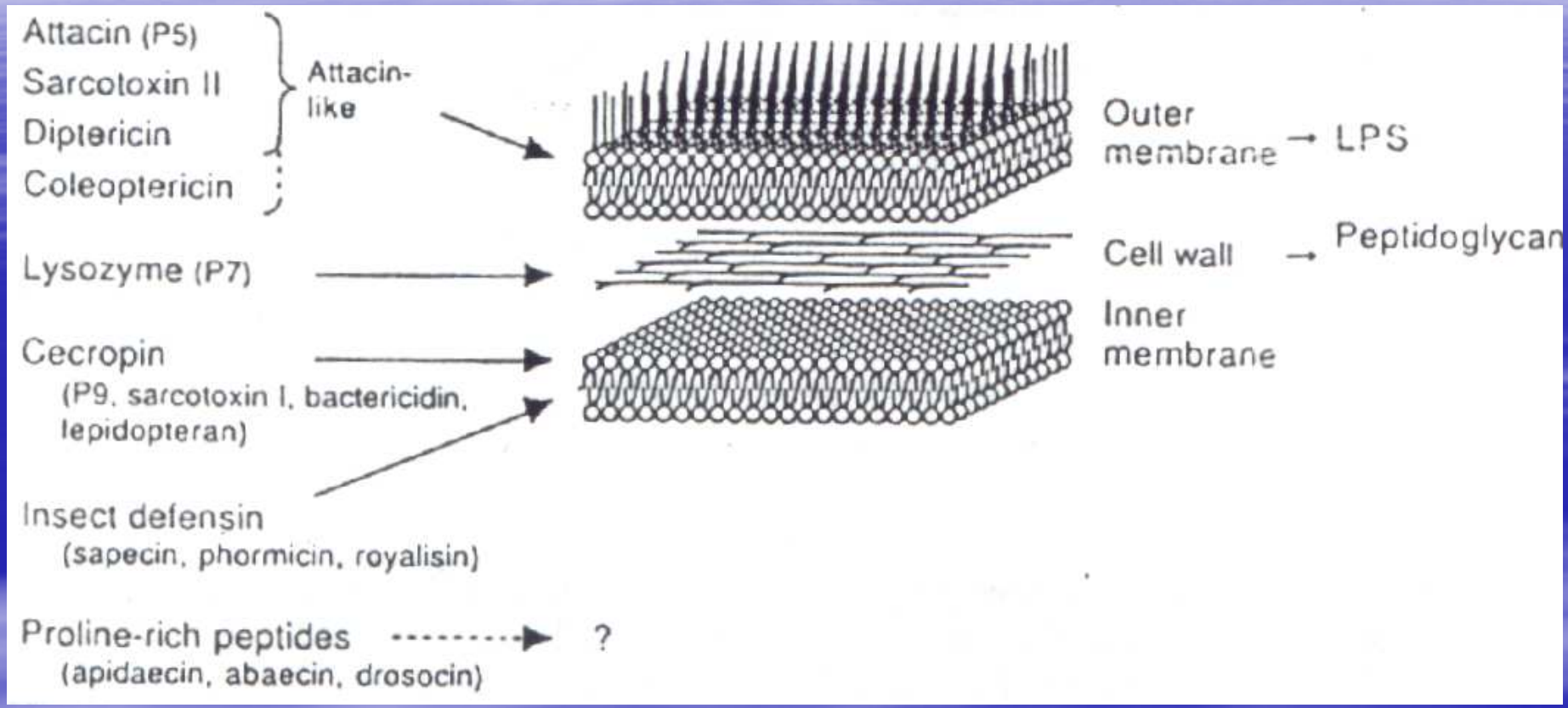
- prohemocyty
- plasmatocyty
- granulocyty
- spherulocyty
- oenocyty

Imunitní reakce hemocytů:

- fagocytóza
- enkapsulace
- nodulace
- koagulace

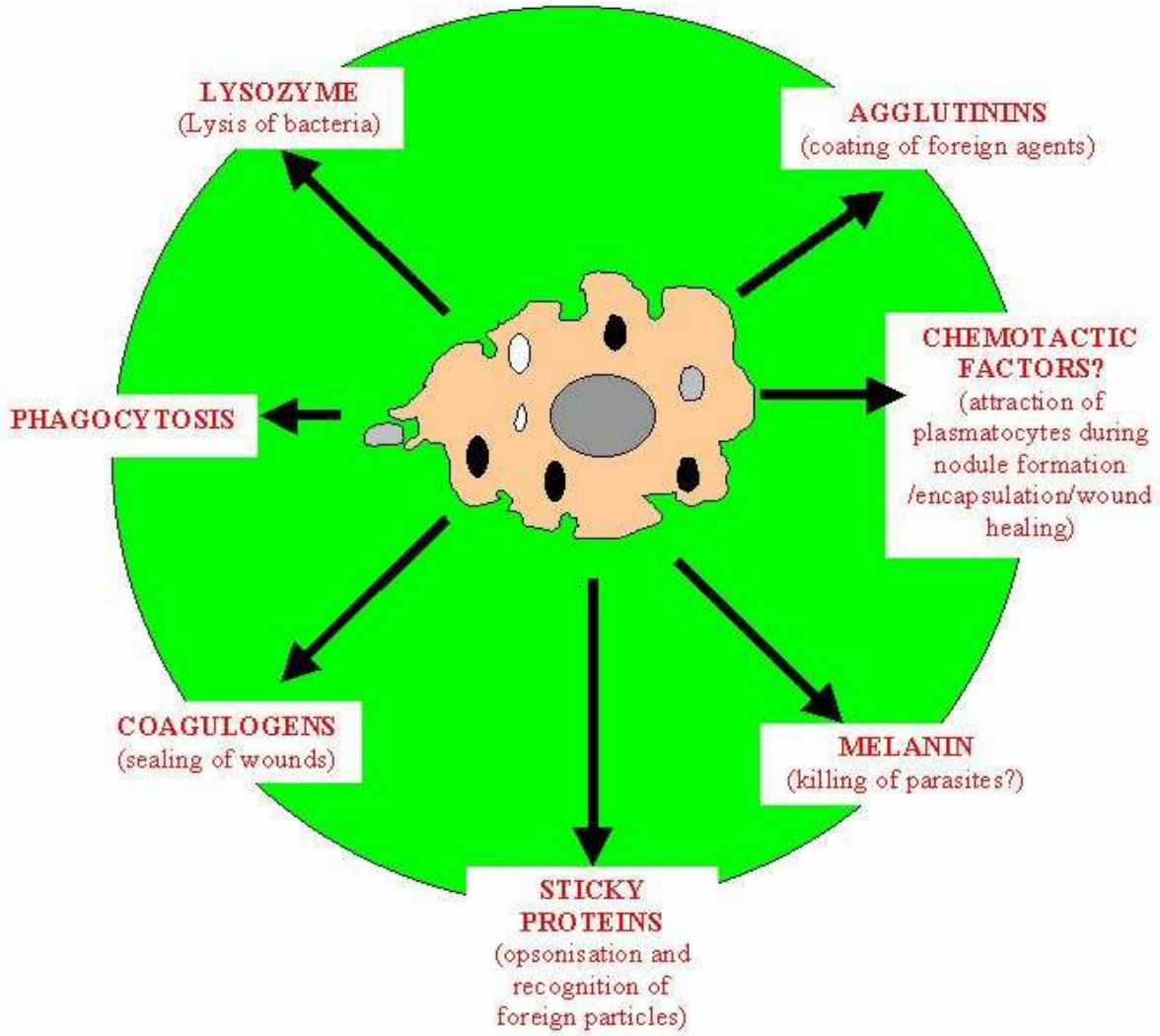


podle Turner 1994



Obr. 1 : Navržené schema působení indukovaných antibakteriálních proteinů a peptidů na buněčnou stěnu bakterií (Hultmark 1993)

Figure 5: Diagram of an insect granular cell emphasizing its multifunctional role.



Elektroforéza SDS – PAGGE

(**s**odium **d**odecyl**s**ulfat **p**oly**a**crylamide **g**radient **g**el **e**lectrophoresis)

- gely vznikají polymerací akrylamidu a N',N'-methylenbisakrylamidu
- SDS (sodium dodecylsulfát) se váže shodně na všechny proteiny v poměru 1,4 g / 1g proteinu a předává jim silný záporný náboj
- mercaptoethanol - rozštěpí disulfidické můstky v molekulách proteinů
- vertikální elektroforéza SE 600 (Hoefer Scientific Instruments)
- 5 % koncentrační gel a separační gel (14 x 10,5 cm) s gradientem akrylamidu 7,5 – 20,0 %
- dělení proteinů probíhalo ve dvou gelech, každý z nich pro 15 vzorků
- standard (SDS-PAGE Molecular Weight Standard Broad Range, Bio-Rad; 6,5 - 200 kDa)
- gely byly barveny stříbrem podle Kirkeby et al. (1993)


UNIVERZITA BRNO
TRG 3
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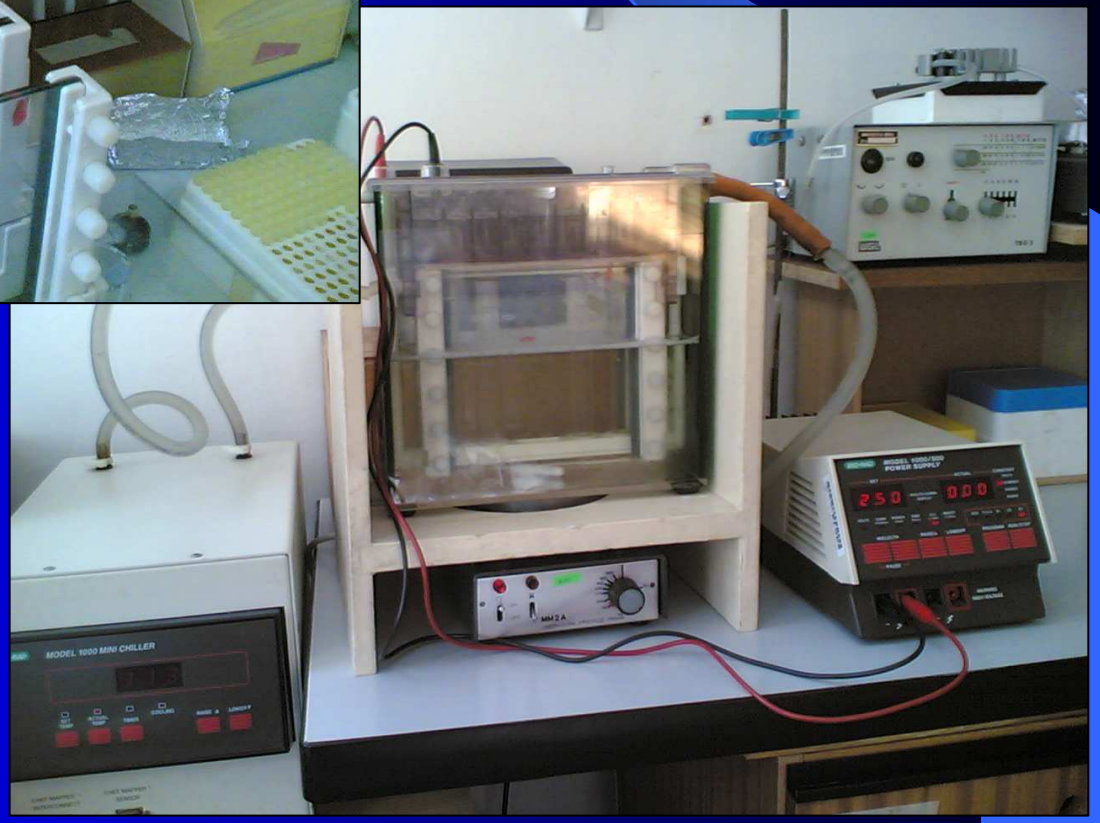
MM 2A


MODEL 1000 MINI CHILLER
MM 2A
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Vyhodnocování gelů:

- videodensitometr GS-670 a software Molecular Analyst (Bio-Rad)

Profilová analýza:

- **kvalitativní:**

  MW jednotlivých proteinových frakcí hemolymfy

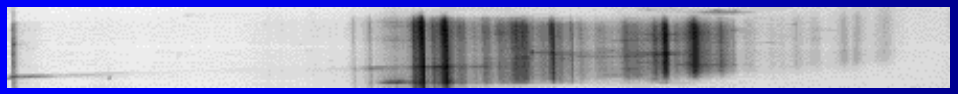
- **kvantitativní:**

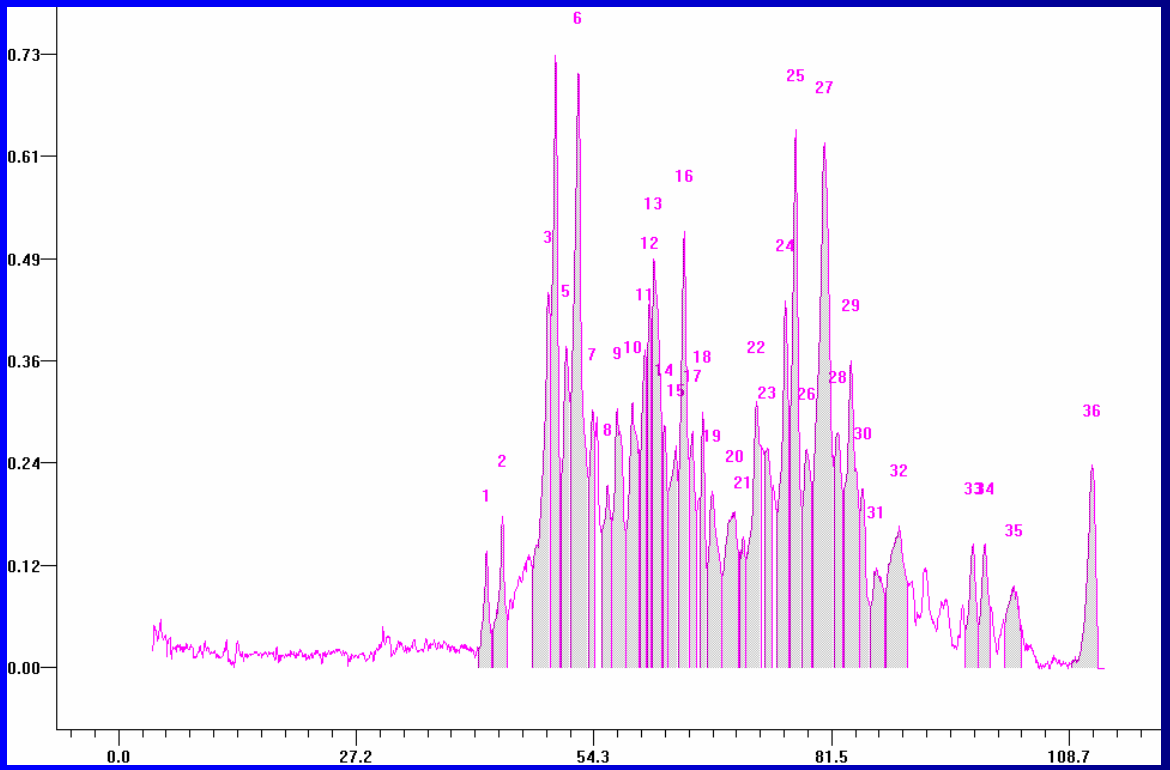
  poměr zastoupení jednotlivých proteinových frakcí

  mezi samci a samicemi během vývoje

Profilová analýza:

- rozdělené vzorky na polyakrylamidovém gelu
- absorbční křivka (osa x – vzdálenost od startu, osa y – optická hustota)
- tabulka s určenou molekulovou hmotností frakcí (MW) a plochou píku





Peak No.	Area (OD*mm)	MW (Da)
1	0.1031	84013
2	0.1521	79290
3	0.4767	66371
4	0.5198	64548
5	0.3230	61972
6	0.8325	59245
7	0.1905	56035
8	0.2044	52885
9	0.3650	50993
10	0.3637	48126
11	0.2294	45910
12	0.1872	45130
13	0.4452	44363
14	0.1340	42593
15	0.2568	40719
16	0.4203	39515
17	0.1859	38265
18	0.1684	36739

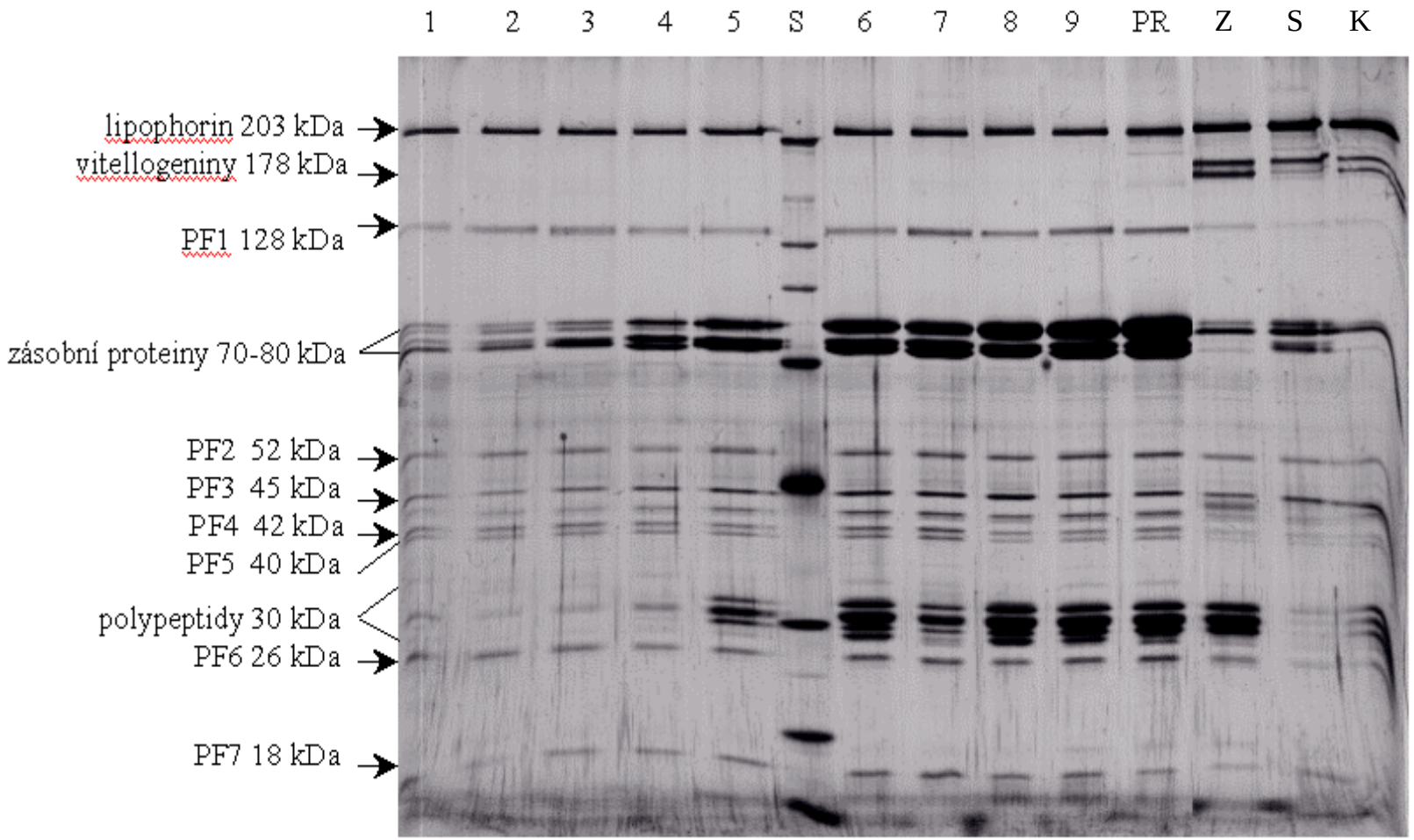
Proteinové spektrum hemolymfy bource morušového (*Bombyx mori*)

Bombyx mori (Lepidoptera, Bombycidae)

bulharský monovoltinní hybrid AS x KK

Bombyx mori – samice

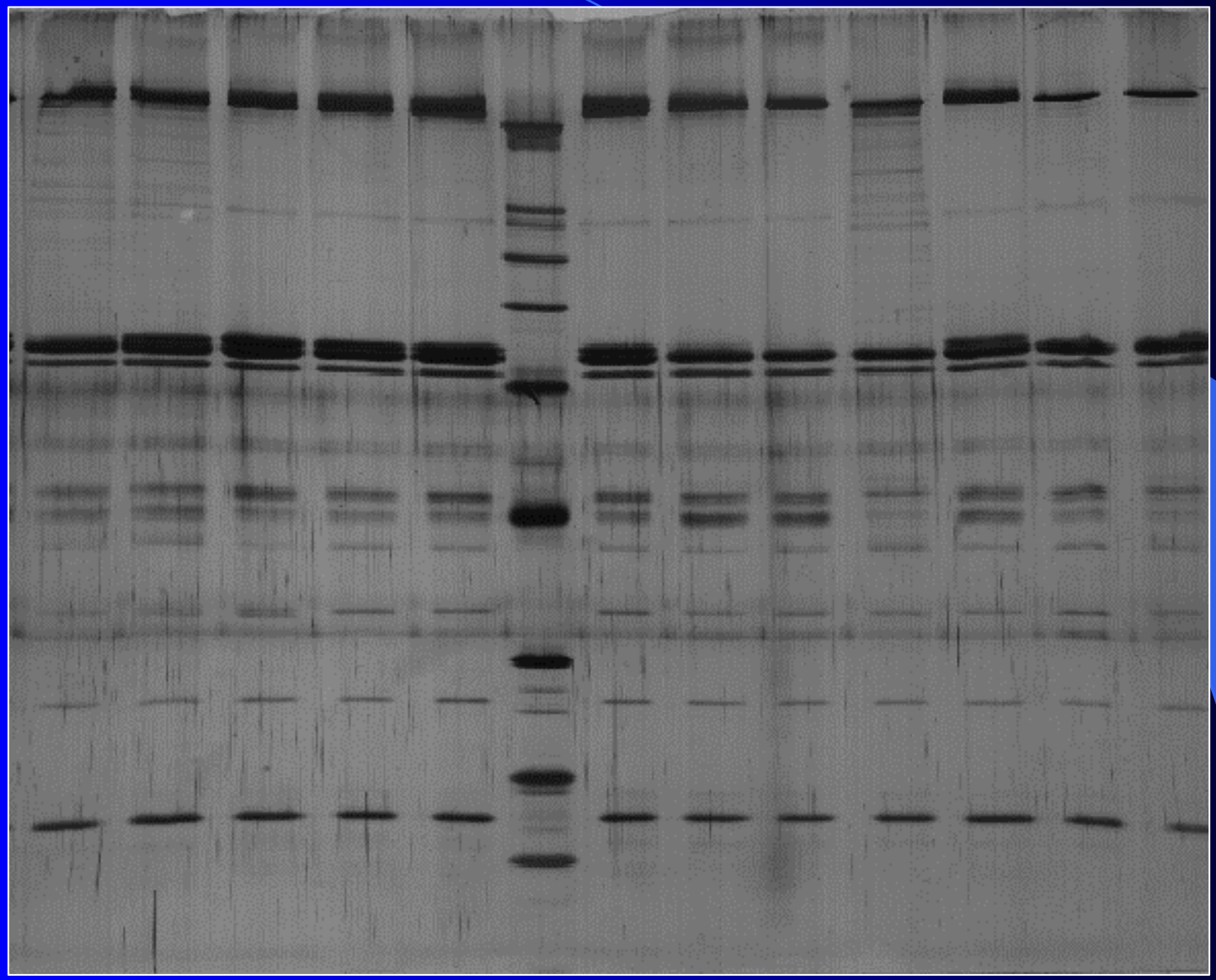
S – standard; 1. - 9. den V. instaru; PR – prepupa 1. den;
Z – kukla začátek; S – kukla střed; K – kukla konec.



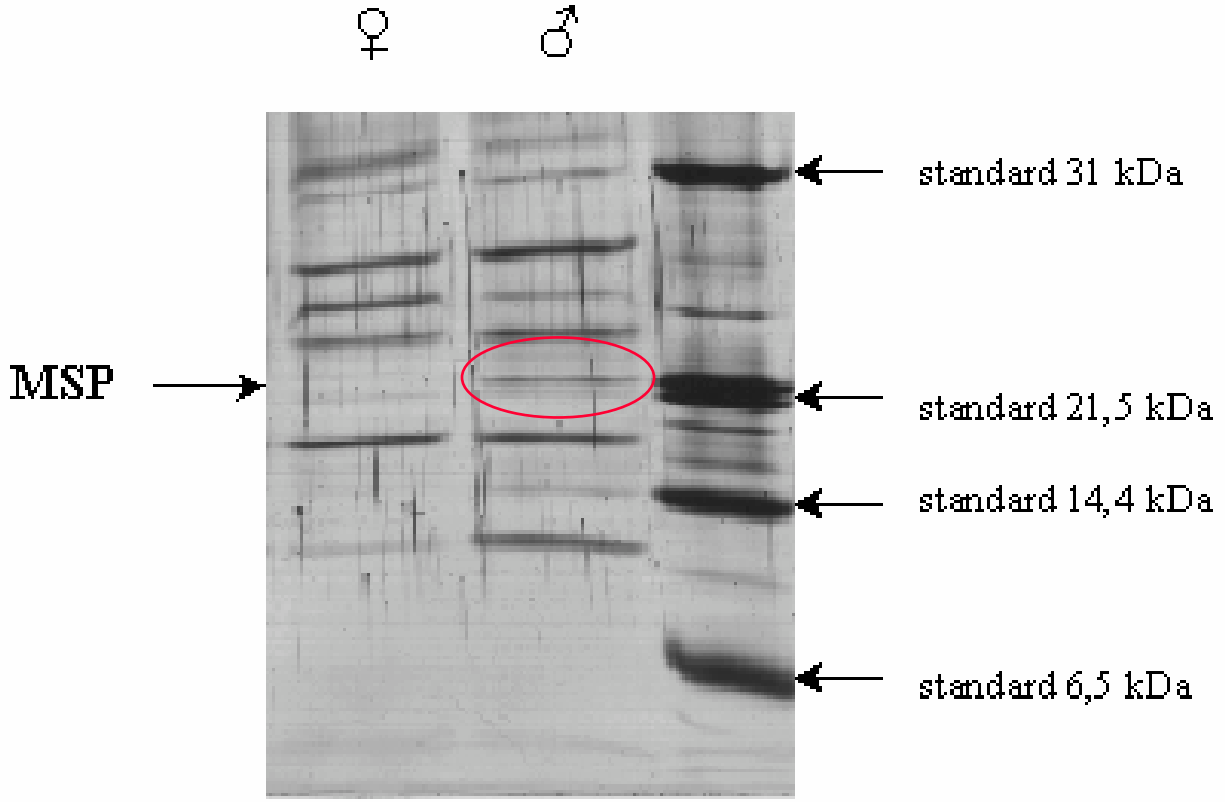
Proteinové spektrum hemolymfy zavíječe voskového (*Galleria mellonella*)

Galleria mellonella (Lepidoptera, Pyralidae)

Galleria mellonella - housenky VII. instaru



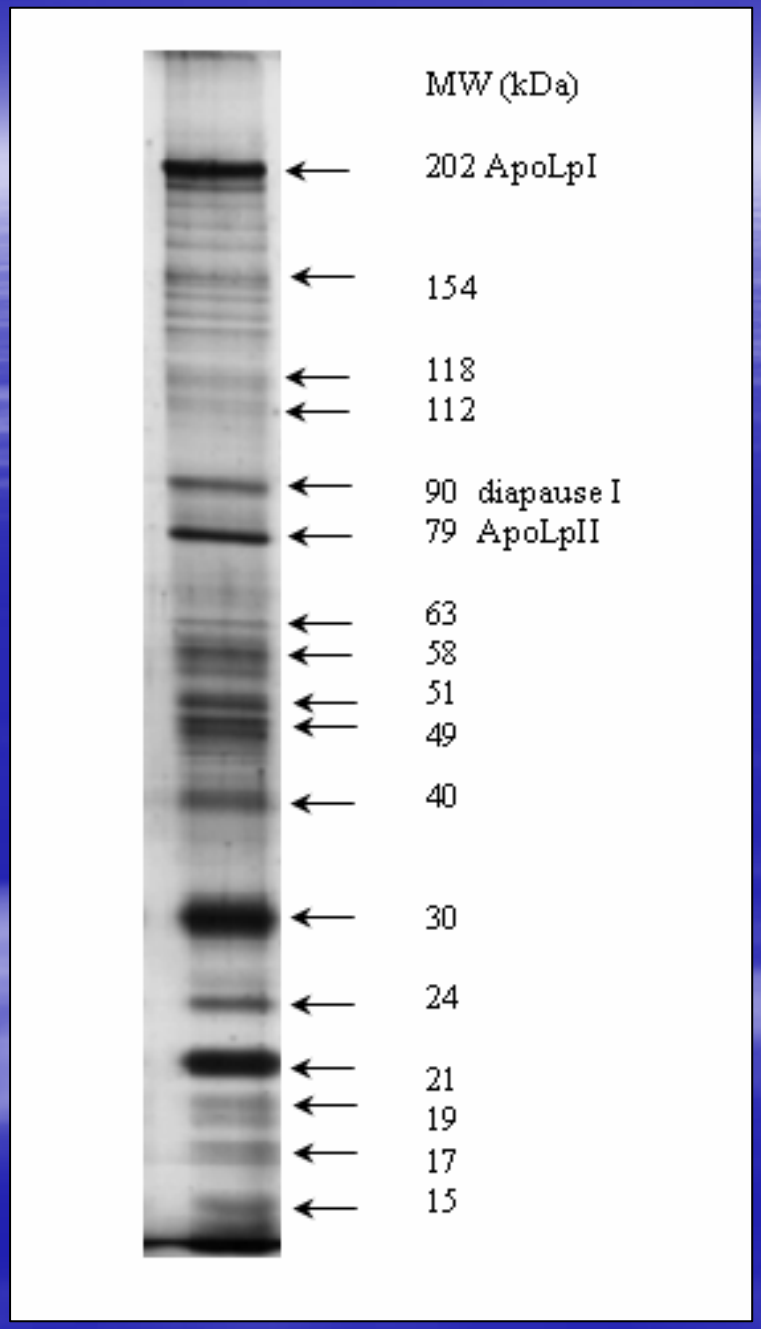
- v hemolymfě starých kukel (tmavé oči) byl detekován male specific protein (MSP), popsaný r. 1998 (Lee et al.)



Proteinové spektrum hemolymfy mandelinky bramborové (*Leptinotarsa decemlineata*)

Leptinotarsa decemlineata (Coleoptera, Chrysomelidae)

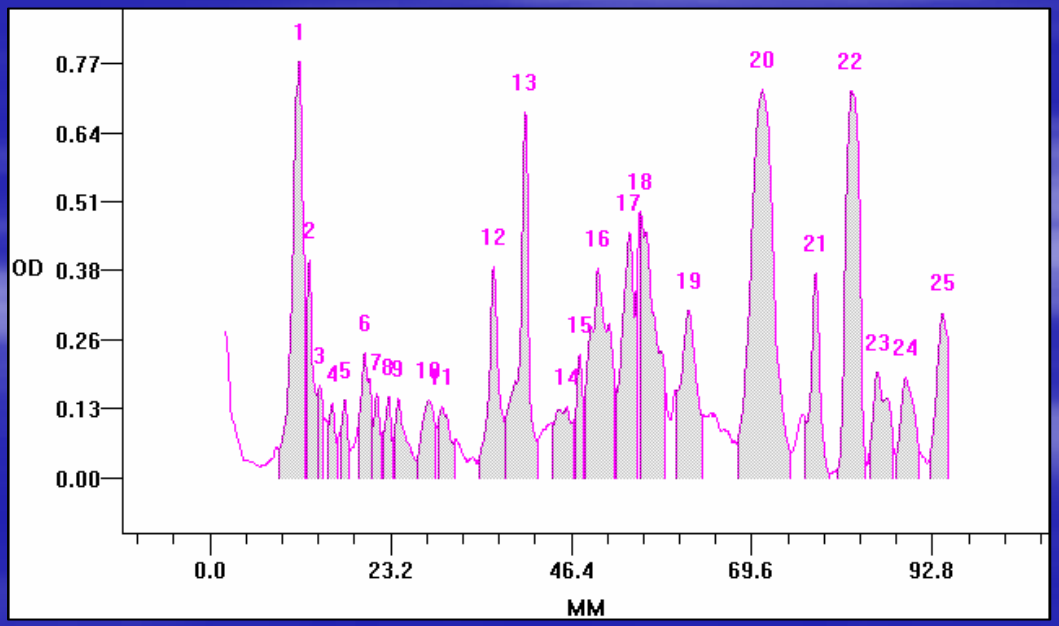
- 36 samic a 35 samců jako potomstvo 18 různých samic
- odběr hemolymfy - 5 dní stará imaga



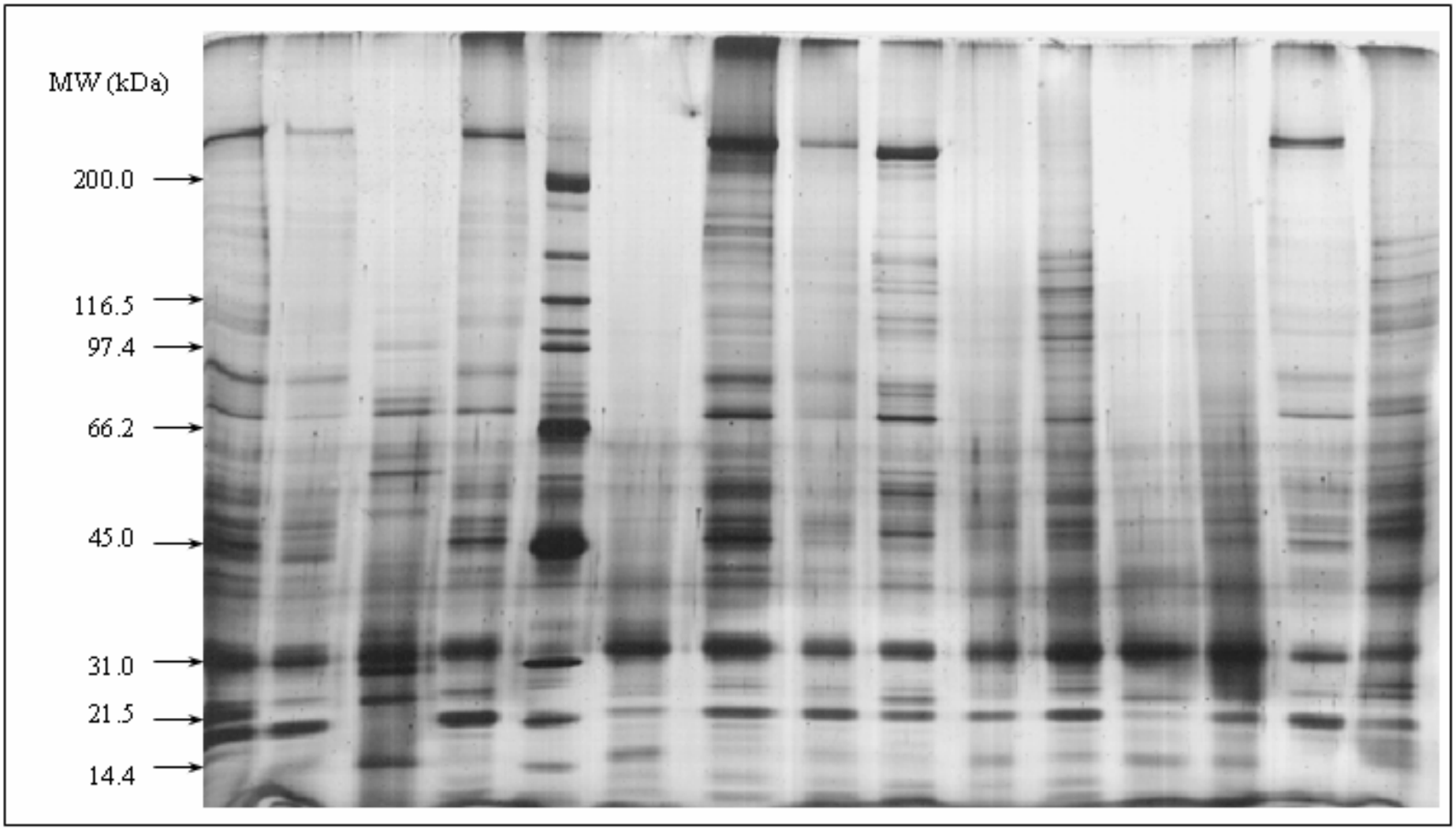
- celkem byla hemolymfa *L. decemlineata* rozdělena na 25 proteinových frakcí v rozmezí 6,5 - 202 kDa

- lipophoriny

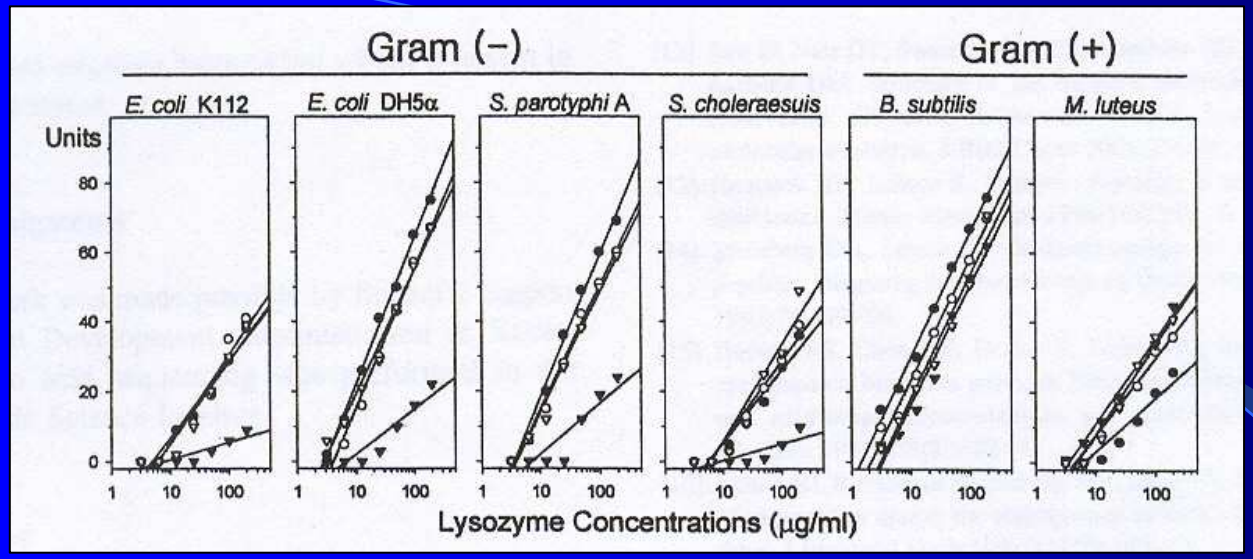
- zásobní proteiny

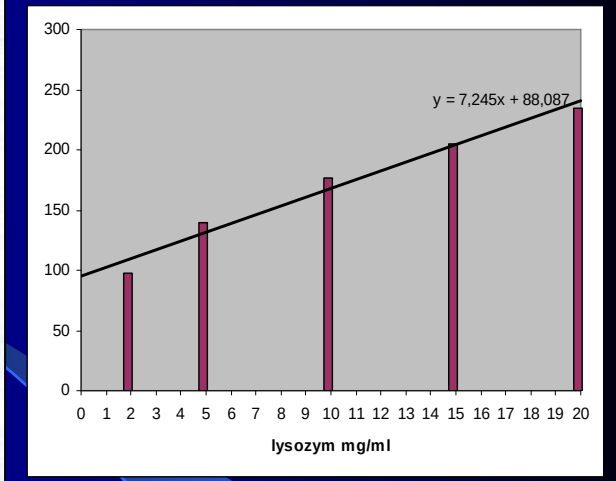


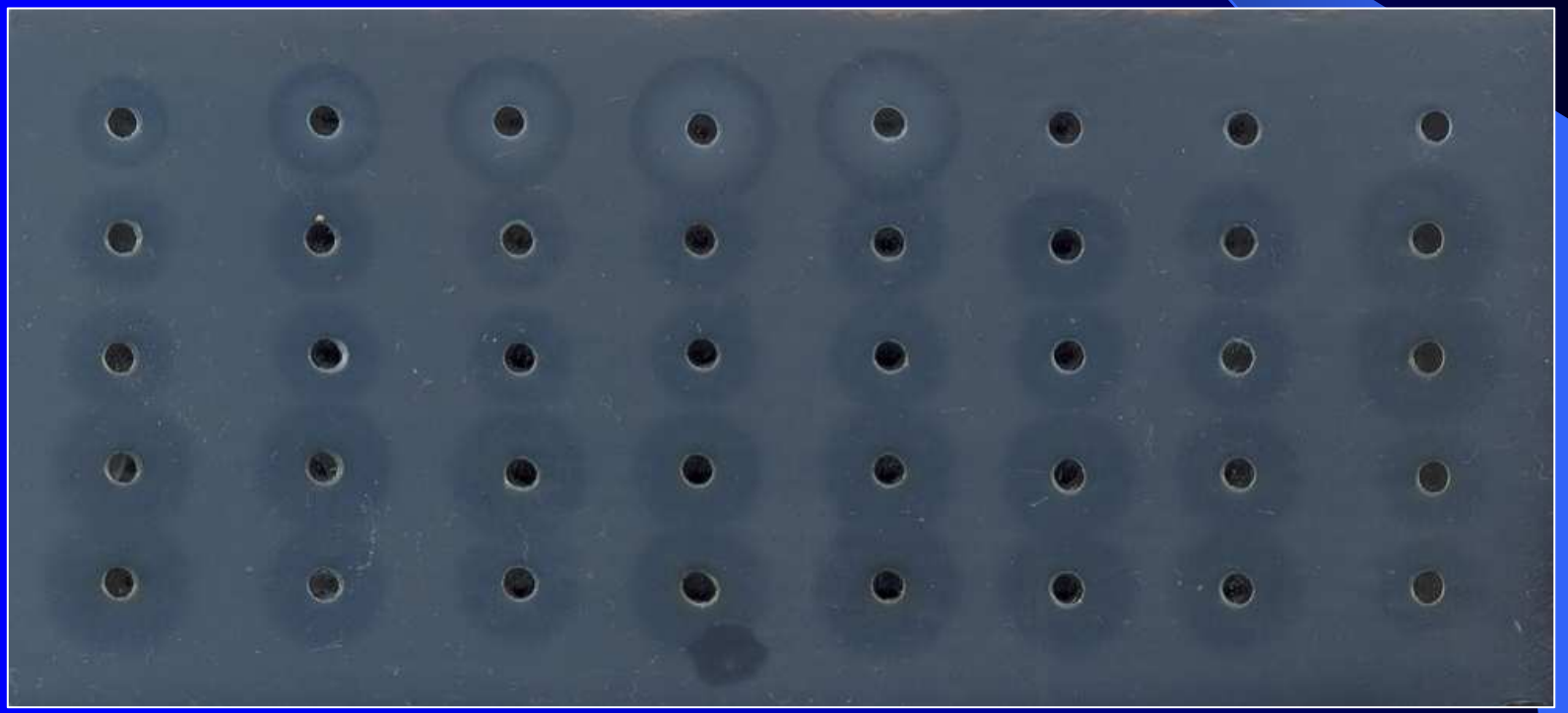
variabilita v populaci – každý vzorek je potomek jiné samice



Aktivita lysozymu v hemolymfě







radíální difúze v agaróze

Entomopatogenní hlístovky (EPN)

třída: Nematoda (Nematoda)

řád: Háďata (Anguilata)

čeleď: *Steinernematidae*

Heterorhabditidae

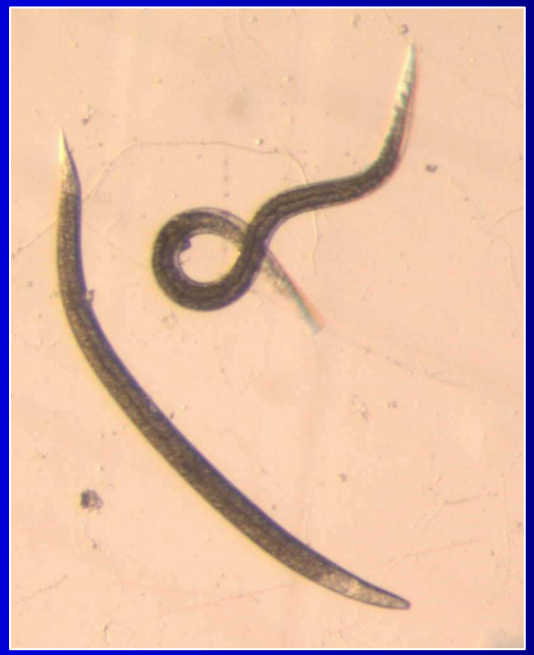
- vyskytují se volně v půdě
- selektivita - výhradně entomopatogenní
- nízké teplotní optimum - do 20°C
- využívají se jako prostředek biologického boje


dospělci
invazní L3 larvy

Detekce enkapsulace EPN u *G. mellonella*, *Amphimallon* sp. a *Hoplia* sp.:

1. *Heterorhabditis bacteriophora*
2. *Steinernema glaseri*
3. *Steinernema scarabaei*

DJ - *H. bacteriophora*



DJ - *S. glaseri*

DJ - *S. scarabaei*

1. *Galleria mellonella* (Lepidoptera, Pyralidae)

2. *Amphimallon* sp. (Coleoptera, Scarabaeidae)

METODY

1. Přirozená invaze EPN:

- larvy *G. mellonella* nebo larvy *Amphimallon* sp., *Hoplia* sp. + 25 EPN, 10% vlhkost
- inkubace 20 – 24 hodin za laboratorní teploty
- celkem bylo použito 70 ks *G. mellonella*, 23 ks *Amphimallon* sp. a 23 ks *Hoplia* sp.

Enkapsulace byla detekována u *G. mellonella* po přirozené invazi *H.bacteriophora*

Shrnutí:

Enkapsulace: ANO / NE

A / přirozená invaze

	G. mellonella	*Amphimallon* sp.	*Hoplia* sp.
H. bacteriophora	**ANO**	NE	NE
S. glaseri	NE	NE	NE
S. scarabaei	NE	NE	NE

B / injikace EPN

	G. mellonella	*Amphimallon* sp.	*Hoplia* sp.
H. bacteriophora	**ANO**	**ANO**	NE
S. glaseri	NE	NE	NE
S. scarabaei	NE	**ANO**	NE

Témata bakalářských a diplomových prací týkajících se entomopatogenních hlístovek jsou zaměřena na:

- testy patogenicity jednotlivých EPN
- srovnání invaze EPN do hostitele v různých prostředích
- obrana hostitele vůči EPN
- možnosti biologického boje s hmyzími škůdci

Výsledkem jsou:

- bakalářské a diplomové práce
- postery a přednášky na konferencích
- publikace

Folia Microbiol. 49 (3), 315–319 (2004) http://www.biomed.cas.cz/mbu/folia/

Silkworm (*Bombyx mori*) Hemocytes Do Not Produce Reactive Oxygen Metabolites As a Part of Defense Mechanisms

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ABSTRACT. To investigate whether hemocytes of *Bombyx mori* (*Lepidoptera*) larvae produce reactive oxygen species (ROS) as part of the oxidative killing of invading pathogens, the production of ROS was measured as a luminol- and lucigenin-enhanced chemiluminescence of unstimulated or stimulated (zymosan particles, phorbol myristate acetate, calcium ionophore, rice starch or *Xenorhabdus nematophila*) hemolymph. No detectable ROS production was found. The spontaneous and activated ROS production measured with hemocytes, *i.e.* under the conditions when the antioxidative potential of hemolymph plasma was eliminated, was again undetectable. Likewise, ROS production by isolated hemocytes was observed by spectrophotometric (NBT test, cytochrome *c* assay) and fluorimetric (using dihydrorhodamine and hydroethidine probes) methods. Hence none of the experimental approaches used indicated the production of ROS by hemocytes of *B. mori* larvae as part of their immune response.

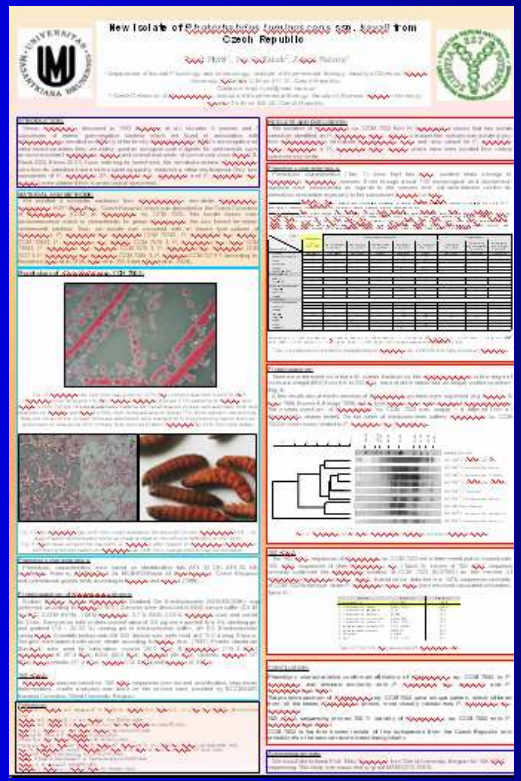
Hemocytes are basic to the invertebrate innate immune system that is divided into cellular and humoral defense responses. The most common types of hemocytes reported in the literature are prohemocytes, plasmatocytes, granulocytes, spherulocytes and enocytes (Yamashita and Iwabuchi 2001; Lavine and Strand 2002). These have been identified by their morphology and histochemical and functional reactions (*e.g.*, Gardiner and Strand 1999). Four basic types of hemocyte immune reactions have been described: phagocytosis, encapsulation, nodulation and coagulation. These activities are always connected with a particular type of hemocytes. During phagocytosis, plasmatocytes and granulocytes are mainly activated while other types of hemocytes have mostly no possibility to phagocytose. Mechanisms participating in the recognition of foreign material are still under study (Lavine and Strand 2002).

The production of reactive oxygen species (ROS) seems to be an important microbicidal factor in both invertebrate hemocytes and vertebrate phagocytes since an increase in ROS production by activated hemocytes of some invertebrates such as *Bivalvia*, *Clitellata*, *Malacostraca*, *Arachnida*, *Echinoidea* or *Ascidiacea* has been reported (*e.g.*, Nakamura *et al.* 1985; Ito *et al.* 1992; Bell and Smith 1993; Valembois and Lassegues 1995; Lambert and Nicolas 1998; Ordas *et al.* 2000; Pereira *et al.* 2001; Azumi *et al.* 2002). However, reports about similar mechanisms in insects are controversial. The aim of the present study was to investigate whether stimulated hemocytes of *Bombyx mori* can produce ROS as part of oxidative killing invading pathogens. Understanding of the defense mechanisms of *B. mori* has a high significance for providing a better insight into the evolution of animal immune systems; it also has a great impact on silk production sericulture.

MATERIALS AND METHODS

Sample preparation. The hemolymph of the Japan polyvoltinne NO2 × CO2 hybrid of the silkworm, *Bombyx mori* LINNÉ 1758 (*Lepidoptera*, *Bombycidae*), was obtained from larvae of the 5th instar. Larvae were reared on mulberry leaves (*Morus alba*) *ad libitum*. The sex was not determined. After the collection of the hemolymph from the first-pair proleg (approximately 150 μL per larva), phenylthiourea was added to protect the hemolymph from melanization. (It was verified that this agent does not have a significant effect

*Corresponding author.



New Isolate of Photobacterium luminescens sp. nov. from Czech Republic

Luminescence 2004; 19: 61–63
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/bio.755

ORIGINAL RESEARCH

Chemiluminescence of lucigenin is dependent on experimental conditions

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ABSTRACT: The aim of the study was to test the effect of experimental conditions such as light radiation and temperature on chemiluminescence (CL) of 10^{-5}–10^{-3} mol/L lucigenin dissolved in various types of solvents. Irradiation by UV light (280, 297, 313 or 400 nm) induced a significant increase in CL of lucigenin dissolved in borate buffer. This effect was the most obvious for 10^{-5}–10^{-3} mol/L lucigenin. All wavelengths used had a similar effect. UV irradiation did not induce changes in the CL activity of lucigenin dissolved in H_2O or in dimethyl sulphoxide (DMSO). Different results for various solvents were not dependent on pH. The CL activity of 10^{-5} mol/L and 10^{-3} mol/L lucigenin dissolved in borate buffer increased depending on the solution temperature (25°C, 30°C, 37°C or 40°C) already at the beginning of the analysis, with a further increase during 16 h incubation period. It can be summarized that temperatures higher than 25°C and intensive light irradiation are among those factors which significantly affect the result of analysis when lucigenin is used as a luminophor. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: chemiluminescence; lucigenin; light irradiation; temperature

INTRODUCTION

Lucigenin has frequently been used in the luminescent detection of superoxide anion radical ($O_2^{\cdot-}$) production by various enzymatic and cellular systems (1–3). The three key steps for detecting $O_2^{\cdot-}$ are: (a) one electron reduction of lucigenin (Luc^{2+}) to cation radical ($LucH^{\cdot+}$); (b) reaction with $O_2^{\cdot-}$ to yield lucigenin dioxetane; and (c) the decomposition of lucigenin dioxetane to electronically excited N-methylacridone which emits photons when relaxed to ground state (1).

The auto-oxidation of $LucH^{\cdot+}$ with a simultaneous production of $O_2^{\cdot-}$ can be a complicating factor (3, 4) and thus experimental conditions can significantly affect the amount of $O_2^{\cdot-}$ detected. We observed an unexpected excitation of lucigenin in various types of previous experiments (see Results and Discussion). Therefore, the aim of this study was to test the effect of experimental conditions, such as temperature and light radiation, on chemiluminescence (CL) of lucigenin dissolved in various types of solvent.

MATERIALS AND METHODS

Reagents

Solutions of 10^{-5}–10^{-3} mol/L lucigenin (bis-N-methylacridinium nitrate, Sigma) were prepared in three types of solvents: sodium borate buffer, pH 9.0 (1.24 g H_3BO_3 and 7.63 g $Na_2B_4O_7{\cdot}10H_2O$ in 500 mL distilled water, both chemicals from Fluka), dimethyl sulphoxide (DMSO, Sigma) or distilled H_2O. All solutions were stored at –30°C before use. All chemicals used were of analytical grade.

Chemiluminescence assay

Lucigenin solutions or solvents alone were pre-incubated for 15 min to raise them to the required temperature (25°C, 30°C, 37°C or 40°C). CL emission of samples (reaction volume 200 μL) was measured in triplicates in 10 min intervals for 16 h using BioOrbit 1251 luminometer (Bio-Orbit, Finland). The data are presented in mV.

The effect of radiation

To test the effect of radiation, 1 mL 10^{-3} mol/L lucigenin was irradiated in plastic dishes at room temperature with UV light (280, 297, 313 and 400 nm) generated by the Oriel Solar Simulator for 10 min, the dose was

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E-mail: alojek@ibp.cz
Contract/grant sponsor: Grant Agency of the Czech Republic; Contract/grant number: 524/02/0095.

Spolupráce:

- Biofyzikální ústav AV ČR (Brno, Laboratoř patofyziologie volných radikálů – měření RKM, fagocytóza)
- Výzkumný ústav včelařství a hedvábnictví (Dol)
- Entomologický ústav AV ČR (České Budějovice, oddělení Patologie hmyzu, Fyziologie hmyzu – antioxidační enzymy…)
- Ústavem zoologie SAV (Bratislava, oddělení Entomologické)
- problematika entomopatogenních hlístovek je napojena na evropský program COST 850

- Socrates Erasmus: Turku, Bari, Zonguldak