中国蚕学会第四届青年学术研讨会
会议论文集

主办单位：中国蚕学会

承办单位：华南农业大学动物科学学院蚕丝科学系

广东省农业科学院蚕业与农产品加工研究所

广东省蚕学会

中国　　广州

2004 年 11 月

中国蚕学会第四届青年学术研讨会
会议论文集

主办单位：中国蚕学会

承办单位：华南农业大学动物科学学院蚕丝科学系

广东省农业科学院蚕业与农产品加工研究所

广东省蚕学会

中国　广州

2004 年 11 月

《中国蚕学会第四届青年学术研讨会会议论文集》勘误

页码/行	误	正	页码/行	误	正
3 倒 11	cause	cause 0.01	222 顺 13	精于	精子
5 顺 2-5	(位置)	(移至 12 行后)	223 顺 4	温力	通力
9 顺 2	ニ-	β-	223 顺 20	许多下子学者	许多学者
58 倒15-16	(作者单位)	Wuxi Agricultural Bureau, 214023	275 顺 12	55-60□	55-60°C
59 倒 13	3×106	3×10^6	301 倒 9	(格式)	(退后两格)
71 倒 6	BmCPV 中株	BmCPV 中国株	322 顺 9	0—4	0—4℃
73 倒 1	for 5h pET286	(pET286 另起段)	324 顺 6	□一□	Ⅰ一Ⅷ
84 倒 4	2×106	2×10^6	324 倒 5	□，……□	Ⅰ，……Ⅰ
85 顺3、7	(段落)	(不另起段落)	324 倒 4	□，□,……	分别为 Ⅰ ⅡⅢⅣ ⅥⅦⅧ
87 顺 14	(段落)	(不另起段落)	327 顺 13	O.2	0．2
98 顺 18	(格式)	(退后两格)	354 顺 3	(段落)	(不另起段落)
104 顺 19	导人	导入	354 倒 14	接着……	另起段落
105 倒 2	注射人	注射入	401 顺 18	（1.2……）	(字体不加粗)
107 顺 12	黄海×苏春	黄海×苏春、	407 顺18-19	(空行)	(无空行)
125	ニ	°C	409 顺 21	(格式)	(退后两格)
143 倒 5	-20ニ	-20°C	411 顺 8	(格式)	(退后两格)
151 倒 9	3 1	3:1	416 倒 10	治疗 组	治疗Ⅰ组
153	ニ	°C	倒 8	治疗 组	治疗Ⅱ组
195 倒 13	*Xba*S和ニ *al*_	*Xba* Ⅰ和 S*al* Ⅰ	428 倒 11	崎报道	岡崎报道
倒 3，8	ニ	°C	429	K+	K^+
196 顺 3	2.4	2.5	445 顺 9	(格式)	(退后两格)
201 顺 18	(作者标号)	(上标)	473	□	°C
206 摘要	■	’	483 倒 4	(格式)	(退后两格)
207 顺 2	片断	片段	487 顺 16	(格式)	(退后两格)
207 顺 4	片断	片段	508 顺 22	(格式)	(退后两格)
207 顺 8	BLAS	BLAST	25	(格式)	(退后两格)
210 顺 15	5	5’	509 顺 8	PH	pH
16，17	5	5’			
210 倒 13	5-UTR	5’-UTR	特别说明：		
	3-UTR	3’-UTR	（1）164-165 页因排版错漏，图片未能印刷出来。		
210 倒 12	3-UTR	3’-UTR			
210 倒9.8	5	5’	（2）279 页图 3 位于序列下方。遮盖了部分序列。		
219 倒 5	ECORI	EcoRI			
	PSC101	pSC101	以原作者会议宣读论文为准，特此致歉。		
倒 4	ECORI	EcoRI			
221 顺 12	86 16	86:16			
倒 12	素才	素材			
倒 7	（1936）年	（1936 年）			
倒 4	精于	精子			

中国蚕学会第四届青年学术研讨会日程安排

11月4日　报到：华南农业大学专家楼；　中午、晚餐　在华南农业大学校内

11月5日　开幕式、照相、　大会报告　以及分组交流

上午	8：30am —9：00 am	开幕式
	9：00 am —9：30 am	照相
	10：00 am —12：00 am	大会报告
	12：00 am—14：00 PM	午餐
下午	2：00 PM —4：50 PM	大会报告
	5：00PM	参观蚕业研究所，晚饭蚕业研究所

11月6日　上午分组交流报告；下午分组交流报告，总结，会议交流结束。
晚上珠江夜游

上午	8：30AM —12：00AM	分组交流报告
下午	2：00PM — 4：00PM	大会报告
	4：00 PM —4：30PM	大会总结、闭幕
	5：00PM	华南农业大学专家楼出发；　珠江夜游 （6：30PM开始，约8：00PM结束）

11月7日

上午　参观宝桑圆

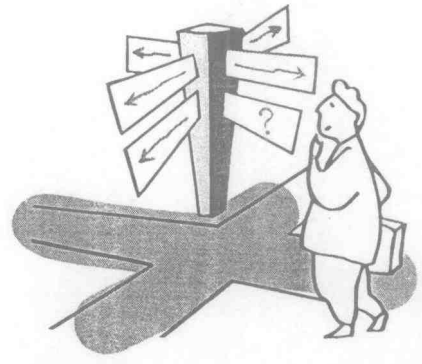
8：30AM—12：00　宝桑圆花都基地（原料）、钟落潭基地（厂）

中午　钟落潭基地午餐　（蚕研所）

下午　回华农大

会议结束；送嘉宾离港

11月8日　早餐后，送嘉宾离港



中国蚕学会第四届青年学术讨论会论文目录 *

（分蚕、桑及其他三类，每类以第一作者姓氏笔画排列）

蚕学类

桑类

蚕业及其它类

西南农业大学论文目录

（按照西南农业大学原始提供的目录排列）

17. 家蚕血液胰凝乳蛋白酶抑制剂的分布调查
18. 主要鳞翅目昆虫胰凝乳蛋白酶抑制剂及其多态性分析
19. Purification, Characterization and clone of hemolymph chymotrypsin inhibitor (CI - 9) of the silkworm, Bombyx mori
20. Analysis of non - special transcription of fibroin P25 through the EST data of Bombyx mori
21. 家蚕转基因干涉载体 GAL4 - UAS 的构建及表达
22. 家蚕气味结合蛋白研究
23. 家蚕 5 龄中期幼虫脂肪体组织基因表达分析
24. Profiling and comparison of gene expression in the silkworm fat body during metamorphosis by EST analysis
25. The Purification and Gene Clone of the Exosporal proteins P30. 4 of N. bombycis
26. Purification, Characterization and clone of hemolymph chymotrypsin inhibitor (CI - 9) of the silkworm, Bombyx mori
27. 家蚕突变基因资源研究的最新进展
28. 家蚕近交系遗传纯度的 RAPD 检测
29. 家蚕保存系统广食性种质的检出与培育
30. 家蚕广食性系统 GS01 对甘蓝摄食性的遗传
31. 家蚕新母性白卵突变的遗传分析
32. The Purification and Gene Clone of the Exosporal proteins P30. 4 of N. bombycis
33. Isolation and purification of novel chitinase inhibitor in pseudomonas sp.
34. The Screening of Bacillus sp. 2108 Which Can Produce the Inhibitor of Bombyx mori Chitinase and the Research of Its Culturing Condition
35. Screening cellulase from metagenomic library
36. A comprehensive survey of transposable elements from Nosema bombycis genomic sequence
37. Survey of Long terminal retrotransposon from Bombyx mori genomic sequence.
38. A SNPs Map of Chongqing Isolate of Bombyx mori Nuclear Polyhedrosis Virus (BmNPV)
39. Studying on mitochondrial pyruvate dehydrogenase complex of Nosema bombycis
40. 家蚕微孢子虫（Nosema bombycis）侵染草地贪夜蛾卵巢细胞（sf21）体系的建立
41. Risk assessment in monitoring horizontal gene transfer in field trials of transgenic plants
42. miRNAs，一种新型的具有重要基因调控作用的小分子 RNA
43. A new densovirus isolated from the silkworm
44. 凋亡细胞的吞噬（engulfment）机制
45. 家蚕晚期胚胎原代细胞分化和转分化的研究
46. 家蚕原代细胞有丝分裂与分化的研究
47. 柞蚕核型多角体病毒（Antheraea pernyi Nucleopolyhedrovirus，ApNPV）lef - 12 基因序列与进化分析
48. 细胞周期检验点调控研究进展
49. 家蚕细胞培养的研究现状及应用
50. 家蚕人工饲料育适应性蚕品种的选育
51. 冷冻保存对家蚕精液乳酸脱氢酶活性的影响
52. 家蚕生殖细胞冷冻保存及精液冷冻生存评价
53. 竹纤维的结构和热性能研究

Studies on Activities of Some Enzymes and Pathology of the Silkworm, *Bombyx mori* Infected with Chinese and Egyptian Flacherie

Li Wenchu[1] Liao Fupin[1] Kulkarni Sarita[2] Huang Yadong[3] Zheng Qing[3] Huang Zhijun[1]

1. Department of Sericulture, College of Animal Science, South China Agricultural University.
2. Brij BugTrap Consultancy Pty. Ltd., Melbourne, Victoria, Australia.
3. Biotechnological Center of Medicine, Jinan University.

510642, Guangzhou, P.R.China.

Abstract The toxicity of Chinese species of flacherie is stronger than that of Egyptian flacherie. The phenol oxidase (PO) activity increased when silkworms were infected with Egyptian flacherie but no significant change was detected with Chinese flacherie. The activities of PO and carboxylesterase CAL in the midgut of infected silkworm gradually increased in the 4th instar (after 3rd day) while it branches away in the 5th instar. The activity of the CAL peaked at the same time when the antibacterial peptide peak which was synthesized by the induction of *E.coli* $K_{12}D_{31}$ in *Antherea pernyi* and *Bombyx mori*. Alkaline phosphatase (ALKP) was actively presents on the inner and outer membrane of silk gland of diseased silkworms. Diaminobenzidine (DAB) method performed on the outer membrane of silk gland from diseased silkworms showed that no cytochrome C and hydrogen peroxide was found on the inner membrane. The NBT/BCIP and DAB method mixed treated fat body showed both reactions could be detected.

Keywords flacherie; *Bombyx mori*; enzymes; silk gland; pathology; insect immunity

INTRODUCTION

Flacherie is the most common silkworm disease that inflicts maximum damage to sericulture industry. The cause of this silkworm disease is mainly due to the pathogen of *Streptococcus bombysis*, which *is* always confused with other pathogens. Earlier studies suggested that flacherie disease could also be caused by cytoplasmic polyhedrosis (CPV), or by pebrine or by flacherie virus (FV). In view of these points bacterial disease formerly called flacherie is more complex and is now classified into four different diseases: Bacterial Flacherie, Septicemia and Sotto (Yao Nan, 1956; Ganga *et al.*, 1991). The outbreak of the epidemic flacherie usually happens when the temperature and the humidity in the rearing room is high and the ventilation is poor (Jameson, 1984), or sometimes if the density of rearing silkworms is high. The low quality of mulberry leaves and low larval physiology causes flacherie easily. The nature of the haemolymph and digestive fluid changes when the larvae are infected with flacherie. When compared with the healthy silkworms the haemolymph of diseased silkworm contains a high percentage of adipohaemocytes and low content of proteins, calcium, magnesium, chloride and the

amount of free amino acids and the refractive index also decreased. The acidity of the hemolymph decreases and the pH reaches 7.0 (Aruga, 1994). In China, flacherie septicaemia is usually caused by *Bacillus spp.* and *Serratia marcescens*. The prevention and cure measurements of NPV and flacheire were studied and carried out earlier in the 1960s with antibiotics. When silkworms were reared with mixed infection of FV and bacteria (Li yongqi *et al.* 1981) the diseased silkworm increased significantly. The pathogens of flacherie were isolated from infected silkworms of Egypt, which included tetrapolyhedron and hexapolyhedron virus and *Bacillus spp.* (Li Wenchu, 2002).

Once the microorganisms invades the silkworms, the prophenoloxidase (proPO, monophenol, L-dopa:O_2 oxidoreductase; E.C.1.14.18.1) activating system switches on immediately to respond for melanin production and immunorecognition (Læonard *et al.* 1985). PO activities in the haemolymph directly reflect the immunoreactions in the beginning of infected silkworms.

Carboxylesterase (E.C.3.1.1.2.CAL) takes a part in the metabolism of fatty acid and proteins. It has a close relationship with the synthesis of antibacterial peptides in fat body and haemocytes.

Infectious flacheire causes digestive disorder and the peristaltic movement in the intestine stops which causes the mulberry leaves to ferment in the intestine. Alkaline phosphatase (ALKP, E.C.3.1.3.1) in the intestine hydrolyses phosphomonoesters of alcoholate and phenol that mainly localize in the midgut, muscles, nerve fibres, malpighian tube and silk glands of *B. mori*. Further, this enzyme relates to the physiological situation of the silkworms and reflects positive membrane transportation of nutrients in the midgut (Eguchi *et al.* 1990). Bacterial infection with *Bacillus thuringiensis* causes direct damage to the midgut tissue of the silkworm with a rapid decline in ALKP activity (Miao, 2002). Diaminobenzidine (DAB) method was mainly performed to detect the activities of Peroxidase (E.C.1.11.1.7), Cytochrome Oxidase (E.C.1.9.3.1) and Catalase (E.C.1.11.1.6). The methods of displaying of enzymes were described in Wen (2001).

This paper describes the activity of CAL, PO in the hemolymph and midgut, and the ALKP and DAB methods in the pathological tissue from infected silkworm with infectious Chinese flacherie and Egyptian flacherie. It is anticipated to make clear of the biochemical changes in the hemolymph, midgut and silk gland of infected silkworm with different species of flacherie in different silkworm rearing countries. The Egyptian and Chinese flacherie referred to *Bacillus* sp. is only described in this paper.

MATERIALS AND METHODS

1. Silkworm Rearing and Infection

The newly hatched silkworm variety-932 was reared in small and labeled containers. Leaves contaminated with Chinese flacherie and Egyptian flacherie of *Bacillus spp.* (10^7~10^8 cells/mL) were isolated and kept in Silkworm Disease of Biotechnology and Molecular Biology, Department of Sericultural Science, College of Animal Science, South China Agricultural University. The contaminated leaves were fed to newly molted 4th instar silkworms. Thereafter the silkworms were reared with high quality mulberry leaves. The control silkworms were fed with distilled water.

Collection of the Samples

Sample collection began on the 2nd day after the infectious mulberry leaves were given.

Haemolymph collection: Every alternate day the diseased silkworms were collected and caudal leg or caudal horn of three silkworms were cut down with shears, and the mixed haemolymph collected in eppendorf tubes on ice bath, except during the molting stage. All samples of

silkworms were sealed with wax and kept on rearing till the silkworms went to death, and instead of another group of three silkworms.

Midgut samples collecting: Midguts of several diseased silkworms were dissected together with undigested mulberry leaves and haemolymph was collected as described above. The samples were then dissolved and homogenized in 500μL of SSC buffer (150mM NaCl and 15mM Sodium Citrate) and centrifuge at 7000 rpm for 10min at 4℃. The supernatant of the samples were examined as original materials.

2. Analysis of Enzymes Activities

2.1 The Extraction of Phenol oxidase (PO) in the Hemolymph Lysate

200μL of hemocytes were collected in equal volume of ice-cold AC-buffer (62mmol/L NaCl, 100mmol/L Glucose, 10mmol/L EDTA, 30mmol/L Trisodium citric dehydrates and Citric acid monohydrate, pH 7.2 , autoclaved) and washed for several times, and the haemocytes were spun at 10,000rpm for 10min. at 4℃ in a refrigerated centrifuge (Centrifuge 5810R, Germany). The resultant cell pellet was resuspended in CAC-buffer (5mmol/L $CaCl_2$, 0.01mmol/L Sodium cacdylate (Sigma), pH7.0, autoclaved) homogenized and centrifuged as above. Or the pellet was lysated and dissolved in 200μL of exam solution (40mm NaOH; 145mmol/L NaCl; 17mmol/L EDTA; 41mmol/L Citric acid; 5% of sucrose; 10mmol/L Guanidine Hydrochloride, pH7.2, autoclaved) .All eppendrof tubes were placed on ice bath, and then assayed for PO activity.

2.2 The Assay of PO Activities

A spectrophotometer (Model-721, Shanghai) was used to assay the PO activities and the methodology was referred to Michael (2001). 200μL of exam solutions were mixed with equal volume of ice-cold substrate solution (1.0 Unit of trypsin (1:250, SABC); 7mmol/L L-dopa (Sigma) in CAC-buffer and spun at 7000rpm for 10min at 4℃. The final volumes were adjusted to 2mL. The activity units of PO is defined at 1μL of haemocytes in exam solution (pH 7.0, 30℃) causes 0.01 absorbance value change at 490nm in 1min as 1unit of enzyme activity.

2.3 The Activities of Carboxylesterase (CAL) and PO of Midgut

The methodology used to assay of activities for CAL was according to Miao (2002) with few modifications. The supernatants of haemolymph and midgut were collected and used as original samples.100mL of incubation buffer (01.mmol/L Tris, pH8.8; 230μL of 10% $MgCl_2$; 2g NaCl; 100mg α-naphtylacetic acid and 100mg fast blue R.R salt was dissolved in 10mL of aceton, and 0.5g polyvinylpyrrolidon (Sigma) (absorbent) was dissolved in 20mL of ethanol and then mixed together and left to standstill for 24hrs and the supernatant was used as incubation buffer. 100μL of sample was added to 900μL of incubation buffer and incubated at room temperature for 25min. 20μL of EDTA was added to end the reactions. The absorbances at 410nm were measured with a spectrophotometer (UV755B, Shanghai). The CAL activities are defined at 1μL of original sample cause absorbance value change in 1min (pH 7.0, 25℃) at 410nm as 1 unit enzyme activity. The activities of PO were measured as described before.

2.4 The Histochemical Display of ALKP and DAB Method in the Tissues under Microscope

The ice-cold sections of fresh tissues preserved in ultra-low refrigerator (-70℃) were incubated with nitroblue tetrazolium/ 5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) dying kit (SABC) or/and diaminobenzidine dying kit (DAB, SABC) in a wet box. The work solution of NBT/BCIP and DAB were diluted accordingly to the user's guidance. The sections were observed and photos were taken under CX-41 phase contrast microscope.

Results

1. The Activities of Phenol Oxidase (PO)

The defense of silkworm consists of both humoral and cellular immunoreactions. Humoral

defense include antibacterial peptides and proteins, reactive intermediates of oxygen or nitrogen and the complex enzymatic cascade. Haemocytes play a very important role in the defense system of insects because haemocytes recognize non-self with molecules on the invading organism or opsonize the surface of the invader such as interaction of lipopolysaccharides and β -1, 3-glucan. There are signal pathways between intercellular and intracellular which then coordinate effective responses (Lavine *et al.*, 2002).

Once microorganisms invade the silkworm, haemocytes immediately react against the invaders. The Pro-PO and PO cascade are the most important enzymes that actively mediate various immunoreactions. Therefore, the activities of PO inflect the function of haemocytes in the infected silkworm. When haemocyte activity is higher the bacterial activity is lower, this in turn

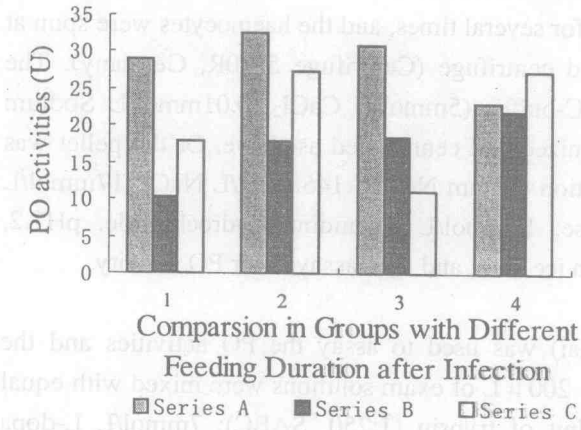


Fig.1 The PO Activities of the Haemocytes Lysate from *Bombyx mori* Infected with Flacherie. Series A indicates the control sample, while B and C indicate the PO activities of the haemocytes lysate infected with the Egyptian flacherie and Chinese flacherie, respectively.

helps cure the silkworm from its disease and get healthy. On the contrary, if the bacterial activity increases the silkworms' disease accelerates eventuating in death.

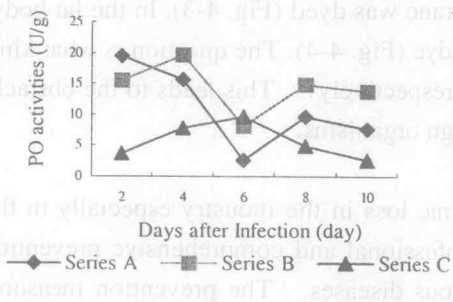
The results of experiments (Fig.1) showed that the PO activity increased steadily when the silkworms were infected with Egyptian flacherie (Series B) while the activity was irregular when infected with Chinese flacherie (Series C). The above results suggest that the toxicity of the Egyptian flacherie species is weaker than that of Chinese flacherie. After 18days of infection there was more than 80% death in the silkworms, which were infected with Chinese flacherie; while less than 20% died when infected with Egyptian flacherie. The toxicity is greatly due to the lab purified and incubated Chinese flacherie, which contains chemical toxin component in the bacterial wall. On the other hand, the silkworms that recovered from the infectious disease show higher activities of PO than that of disinfected (series B).

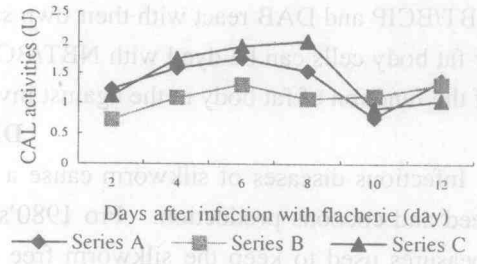
2. Activities of PO and CAL in the midgut

Flacherie disease of silkworm mainly causes the biochemical changes in the midgut and affects the nutrition transport into haemocoel. The activities of PO and CAL in the midgut of diseased silkworms were investigated in this paper. PO activity in the midgut of infected silkworm gradually increased in the 4th instar for 3days; and then gradually decreased in the newly moulted 5th instar. A peak of PO level was present in the 5th instar (Fig 2), but the concentration of PO was much lower than that in 4th instar. The results suggested that bacteria activate the PO cascade in the beginning of infection, which causes a high level of PO in the 4th instar, which then decreases in the 5th instar.

PO activity of the haemolymph collected from the injured silkworms was higher than that of the

silkworms that were not wounded.





The activity of CAL in haemolymph of the infected silkworm gradually increased in the 4th instar and the period after moulting but decreased on the 10th day in the 5th instar. Infected silkworms showed a lower level of CAL activity than the control but the activities increased to a small summit all together on the 8th day. The related changes of PO and CAL activity from 4th

Fig.2 PO Activities of the midgut from silkworm, *Bombyx mori* infected with flacherie.

Fig.3 CAL Activities of the midgut from silkworm, *Bombyx mori* Infected with flacherie.

Series A and B indicate the PO and CAL activities of the midgut infected with Chinese flacherie and Egyptian flacherie in Fig.2 and 3, respectively. While series C indicates the control. X-axis represents day's interval development of silkworm after infection with flacherie from 4th to 5th instar.

instar and 5th insatr suggested that the biochemical changes in the midgut of infected silkworms are consistent in defense system.

3. Histochemical Display of ALKP and DAB Method in the Diseased Silkworm Tissues

In order to study the enzyme activity in the tissues of the silk gland and fat body, the tissues were anatomized with ice-cold dissection machine (Zhejiang, China). The tissue sections were cut using a knife that was maintained at -2° C, while the table temperature was maintained at -30°C to protect the enzyme activities. The inner and outer membrane of silk gland were dyed with NBT/BCIP suggesting that the activity of ALKP was very high in the membrane of silk gland from diseased silkworms (Fig 4-1).

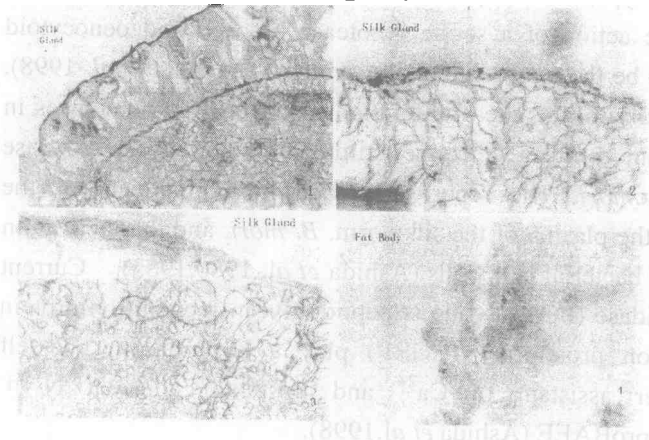


Fig.4 Histochemical Display of NBT/BCIP and DAB Method in Silk Gland and Fat Body of Diseased and healthy Silkworm.

Fig 4-1(100×) showed silk gland from diseased silkworm. Blue arrows indicate the inner and outer membranes of silk gland were dyed with NBT/BCIP ,while Fig 4-2(400×) (CK): just little dying under the same condition (azury arrow indicated); and Fig 4-3(100×): The outer membrane was dyed with DAB method but the inner membrane keeps out of dying (blue and white arrow indicated, respectively). The window on the upper left display enlarged outer membrane of silk gland (1000×). Fig 4-4 (400×) showed NBT/BCIP and DAB mixed dying of healthy fat body. Ice-cold dissection of silkworm: the knife temperature:-02℃; The table temperature:-30℃.

The inner membrane of healthy silkworm was dyed with azure (light blue arrow indicated in

Fig 4-2) under the above conditions. The most interesting result was that the inner membrane of silk gland did not dye with DAB method but outer membrane was dyed (Fig. 4-3). In the fat body, NBT/BCIP and DAB react with their own substrates and dye (Fig. 4-4). The question is what kind of fat body cells can be dyed with NBT/BCIP and DAB respectively? This leads to the obstacle of the function of fat body in the against invasion of foreign organisms.

DISCUSSION

Infectious diseases of silkworm cause a major economic loss in the industry especially in the seed and cocoons production. Fro 1980's there are professional and comprehensive prevention measures used to keep the silkworm free of the infectious diseases. The prevention measures followed are follows: (1) Disinfect all tools and rearing rooms before and after silkworm rearing; (2) Maintain suitable temperature and humidity in the rearing room; (3) Rear silkworms with high quality leaves; (4) Treat moulting silkworm with caution. (5) Separate slow growing and weak silkworm (6) Gather under-grown larvae, (7) Reject diseased silkworm, that control proliferation and transmission of pathogen; (8) improve the characters of diseases resistance by selection of silkworm and (9) Maintain suitable environments in the cocoon spinning stage, such as humidity and air flow when mounting.

Since 1980's research in the field of silkworm diseases has progressed vastly. However a few literatures can be referred in aspect of biochemistry of silkworm flacherie diseases. This paper is the first to report on the enzymatic activities on the inner and outer membrane of silk gland from diseased silkworm, especially the haemolymph and midgut of silkworms infected with flacherie. This paper also reports on the technology of ice-cold sections for the study of the diseased silkworms.

As described before, pathogens cause humoral and cellular immunity response in the beginning of infection. Scientists around the world worked on innate immunity of insects in the recent years. Humoral immunity includes lysozymes, antibacterial peptides (cecropins, proline and glysine rich peptides), defensins, and antifungal peptides. In the fields of cellular immunity, vast amounts of literatures focus on the signal pathway of *Drsophila melangaster*. Lavine *et al* (2002) reviewed the insect hemocytes and their important role in insect immunity. Three kinds of PO (granular, wound, and hemolymph) and a lactcase-type PO are present in insect. It was shown that the zymogen of PO is activated through the action of a serine preotease cascade and oenocytoid hemocytes of silkworm, *Bombyx mori*, to be the major site of PO synthesis (Ashida *et al*. 1998). Therefore, the level of PO activities directly inflicts the proliferation of oenocytoid hemocytes in the cavity of infected silkworms. Serine protease enzyme stimulates the prophenoloxdase activating enzymes (PPAE) of *Bombyx.mori*. The report described the component of the prophenoloxidase cascade are present in the plasma of the silkworm, *B. mori*, and that activation of this enzyme is triggered by elicitors of bacterial cell walls (Ashida *et a*l. 1980,1983). Current knowledge on the proPO and phenol oxidase (PO) cascade is peptidoglycan recognition protein (PGRP) and β -1,3 –glucan recognition protein (β -GRP) play a key role in non-self recognition, and activate PPAE under assistant of Ca^{2+} and upstream adjacent N- α -benzoylarginine ethyl ester (BAEE) and proBAEE (Ashida *et a*l.1998).

The activity of CAL in the midgut was dependent on the stage of moulting, the state of feeding, the qualities of mulberry leaves and temperature. The activity also changes in the different species of silkworm and sex (Miao, 1988; 1989). In this experiment, silkworm reared under the same conditions resulted only in minor sex difference. The activities of CAL peak was present at the same time which coincided with the time of the peak of antibacterial peptide synthesized which was induced with *E.coli* $K_{12}D_{31}$ in *Antherea pernyi* and *Bombyx mori*.

ALKP catalyze every kind of alcohol and phenol and transfer phosphate that is localized on the cell membrane for active transportation. This was consistent with the experiments conducted here, where the active ALKP was present on the inner and outer membrane of silk glands of diseased silkworms than that of healthy silkworms; suggesting that ALKP plays a key role in disease resistance. The last enzyme is cytochrome oxidase in the electronic transfer system of breath assembling on the inner mitochondrium membrane. DAB itself was oxidized in the process of deoxidize cytochrome C and the later was oxidized again by cytochrome A. The circulate reaction proceeded result in DAB continually be oxidized and the product of the reaction precipitated on the reactive part of the tissue. The reaction was detected (Fig 4-4) in fat body. The fig 4-3 demonstrated that the reaction only proceeded on the outer membrane of silk glands in the diseased silkworms and no cytochrome C and hydrogen peroxide on the inner membrane.

SUMMARY

Flacherie disease is one of the main pathogen that causes large economic losses in cocoon and egg production. In the experiment, both Chinese and Egyptian species of flacheries were applied to the silkworm rearing and the results are as follows:

1. The haemolymph from diseased silkworms showed that the phenol oxidase (PO) activities increased when silkworms were infected with Egyptian flacherie but no significant changes were detected with Chinese flacherie. The results indicate toxicity of Chinese flacherie is stronger than that of Egyptian flacherie.

2. PO activity in the midgut of infected silkworm gradually increased in the 4^{th} instar (3days) and newly moulted silkworms in the 5^{th} instar. PO levels were high initially and then gradually decreased. Infectious disease causes the activities of carboxylesterase (CAL) to gradually increase in the 4^{th} instar and the period after moulting, but decrease on the 10^{th} day in the 5^{th} instar. Infected silkworms showed a lower level of CAL activities than the control but the activities increased to a small summit all together on the 8^{th} day. The activities of CAL peak present at the amazing time coincided with the time of the peak of the antibacterial peptides, which was synthesized and induced with *E.coli* $K_{12}D_{31}$ in *Antherea pernyi* and *Bombyx mori*.

3. Histochemical studies showed that the inner and outer membranes of silk glands were dyed with NBT/BCIP. The results suggested that the very activity of ALKP in the membrane of silk glands was from the diseased silkworms that were only in the outer membrane that was dyed with DAB method. It demonstrated the reaction only proceeded on the outer membrane of silk glands in the diseased silkworms and no incidence of cytochrome C and hydrogen peroxide on the inner membrane. NBT/BCIP and DAB methods mixed and treated to the fat body showed that both reactions could be detected.

ACKNOWLEDGEMENT

The research was kindly supported by the Presidential Science Fund of South China Agricultural University, Project No. 2003K023, No. 2003S012 and the National Nature Science Fund of China, Project No. 30170717. The authors sincerely thank Prof. Xu Fengcai for his professional views on enzymes and Prof. Xu Xingyao and Assistant Prof. Zhong Yangjin for their critical opinions on silkworm pathology and anatomical physiology. The above professionals are thanked for their diligent support in all aspects and the authors thank them one and all. Lastly thanks to the editors for accepting and approving the paper.

References

Ashida M and Brey P.T. 1998. Recent advances in research on the insect prophenoloxidase cascade. In "Molecular Mechanisms of Immune Responses in Insects". pp135-172. Edit by Paul T. Brey and Dan Hultmark. Chapman &

Hall, 2-6 Boundary Row, London SE1 8HN.

Ashida M. and Dohke K. 1980.Activation of prophenoloxidase by the activating enzyme of the silkworm, Bombyx mori. Insect Biochem. 10,27-47.

Ashida M., Ishizaki Y., and Iwahana H. 1983.Activation of prophenoloxidase by bacterial cell walls or β-1, 3-glucans in plasma of the silkworm, Bombyx mori. Biochem. Biophys. Res. Commun. 113,562-568.

Aruga Hisao. 1994. Principles of Sericulture (Translated from Japanese). Oxford and IBH Publishing Co.Pvt.Ltd.66 Janpath, New Delhi, 110001.

Eguchi M., Azuma M., Yamamoto H., and Takeda S. 1990.Genetically defined membrane-bound and soluble alkaline phosphatase of the silkworm: their discrete localization and properties. Progre. Clin. Biol. Res. 344:267-287.

Ganga G. and J.Sulochana Chetty. 1991.An Introduction to Sericulture. Oxford and IBH Publishing Co. Pvt.Ltd. 66 Janpath, New Delhi, 110001.

Jameson A.Pringel D. Sc. Report on the Diseases of Silkworm in Indian. 1984. International Books &. Periodicals Supply Service. 24B/5,Deshabandhu Gupta Road, Karol Bagh, New Delhi-110005.

Lavine M.D. and Strand M.R. 2002. Insect haemocytes and their role in immunity. Insect Biochem. Mol. Biol. 32:1295-1309.

Leonard C. Ratcliffe N. A. and Rowley A. F. 1985.The role of prophenoloxidase actiation in non-self recognition and phagocytosis by insect blood cells. J. Insect Physiol. 31(10): 789-799.

Li Rongqi, Lu Xuefang, Ma Dehe. 1981. Studies on the relatioship of virus and bacteria flacherie of silkworm, Bombyx mori. Acta Sericologica Sinica. 7(4): 219-222.

Li Wenchu. 2002. A journey in Egypt: the epidemic situation and prevent methods of silkworm disease. Guangdong Sericulture. 36 (4): 42-45

Miao Yungen, 1988. Studies on the alkaline phosphatase in the midgut of domestical silkworm. Acta Sericologica Sinica. 14(3): 154-158.

Miao Yungen, 1989. Effect of several factors on the alkaline phosphatase in the midgut of silkworm. Bombyx mori. Acta Sericologica Sinica.15(3): 207-211.

Miao Y. -G. 2002. Studies on the activity of the alkaline phosphatase in the midgut of infected silkworm, Bombyx mori L. J. Appl. Ent. 126:138-142.

Yao Nan. 1956.The flacherie questions of Silkworm, Bombyx mori. Bull. Seri. Sci. (in Chinese) 2:46-52.

Wen Jianguo, 2001. Oxidase, in Histochemistry Edited by Bi Chang'en, Li Shugeng. The Publishing House of the People's Health (in Chinese). 358-366.

Transcriptional Activity of *Drosophila hsp70* Promoter Enhanced by the Homologous Region 3 from *Bombyx mori* Nucleopolyhedrovirus in Insect Cells

Shun-Ming Tang, Yong-Zhu Yi, Ya-Jing Zhou, Zhi-Fang Zhang*, Yi-Ren Li and Jia-Lu He

Key Laboratory of Silkworm Biotechnology, Ministry of Agriculture,Sericultural Research Institute, Chinese Academy of Agricultural Sciences,Zhenjiang 212018, China

Abstract *Drosophila melanogaster* heat shock protein 70 gene promoter (D*hsp70p*) is widely used in transgenic insect to drive exogenous gene, and the homologous region 3 from *Bombyx mori* nucleopolyhedrovirus (BmNPV*hr3*) functions as an enhancer for several promoters. To test whether BmNPV*hr3* can enhance the

Shun-Ming Tang, PhD, E-mail: tangshunming2002@163.com
Zhi-Fang Zhang, PhD and Professor, Correspondence Author, E-mail: zjsbsri@public.zj.js.cn

D*hsp70p*'s transcriptional activity, the reporter plasmids, which contain the D*hsp70p*, the reporter, *β-galactosidase* gene with SV40 terminator and BmNPV*hr3* fragment, are constructed and transfected into the insect cell lines (Bm-N cells and Sf-21 cells) by lipofectin-mediated method. The results from the transient expression assay show that BmNPV*hr3* increases significantly D*hsp70p*'s transcriptional activity both under the normal condition and under the heat-shock treatment, although the effects are significantly different between in Bm-N cells and in sf-21 cells. The enhancement way of BmNPV*hr3* on the D*hsp70p* is in an orientation-independent manner. Meanwhile, the effects of heat-shock treatment on D*hsp70p* alone or D*hsp70p*/BmNPV*hr3* combination present no significant difference, indicating that BmNPV*hr3* only enhances the transcriptional activity of D*hsp70p*, but can't alter the characteristic of the response to the heat stress. The above results suggest that the D*hsp70p*/BmNPV*hr3* combination is more effective one to drive exogenous gene for transgene or stable cell expression system in insects.

Key words Heat shock protein 70 gene, Promoter, Enhancer, Insect cell line, BmNPV*hr3*, Transient expression

Introduction

Heat shock protein-70 (Hsp70) belongs to the highly conserved protein class of chaperones (Lindquist *et al.*, 1988; Feder *et al.*, 1999). Heat shock proteins are involved in protecting cells from hyperthermic stress by binding to denatured protein and assisting in exact refolding (Beckmann *et al.*, 1990; Cotto *et al.*, 1999; Feder *et al.*, 1999). Under the normal condition, the expression of *hsp70* is kept at very low level, and increased sharply under the stress conditions, such as heat-shock (Kimura *et al.*, 1999; Uhlirova *et al.*, 2002), hypoxic (Ricchi *et al.*, 2001), chemicals (Hung *et al.*,1998; Zhao *et al.*, 1999), hormone (Lacoste *et al.*, 2001) and electrical stress (Yanagida *et al.*, 2000) etc. Due to the characteristics of inducible ability and lack of organism- and tissue-specificity, *hsp70* promoter (*hsp70p*) is widely used in the transgene and gene therapy to drive exogenous gene (Uhlirova *et al.*, 2002; Schmidt *et al.*, 2004). In order to elevate its transcriptional acitivity for transgene and make it express specifically for gene therapy, many trials had been carried out *in vivo* and *in vitro* (Huynh *et al.*, 1999; Zhao *et al.*, 1999; Lacoste *et al.*, 2001; Schmidt *et al.*, 2004).

Baculovirus homologous regions (*hrs*) are repeated sequences interspersed in the genomes. Up to date, *hrs* have been identified in almost all of baculovirus, including *Bombyx mori* Nucleopolyhedrovirus (BmNPV). There are seven *hrs* in BmNPV T3 genome, that is *hr1*, *hr2L*, *hr2R*, *hr3*, *hr4 L*, *hr4 R* and *hr5* (Gomi *et al.*,1999). Almost all of *hrs* plays an important role in viral DNA replication and functions as an enhancer for some viral and nonviral genes' transcription (Lu *et al.*, 1997; Lo *et al.*, 2002; Viswanathan *et al.*, 2003). We cloned the *hr3* fragment from BmNPV ZJ8 genome (Genbank accession No U51328) and find that it can function as an origin of viral DNA replication and increase the transcriptional activity of viral gene promoter, such as *helicase*, *gp64*, *ie-1*, *egt* (Zhang *et al.*, 1995; Xiao *et al.*, 2001; Zhou *et al.*, 2002, 2003; Shen *et al.*, 2004).

The aim of the present study was to determine whether the BmNPV*hr3* can also enhance the *Drosophila hsp70p's* transcriptional activity using transient expression system in order to obtain a clue as to its possible application in the transgenic insect or stable cell expression system to increase the product of exogenous gene.