

Drug screening and safety test using silkworm as model animal



In spite of its appearance, silkworm takes after human in lots of ways such as analogous tissues or organs, similar sensitivities to pathogens and comparable effects of drugs, and it is low in cost, in little conflict with ethical problem and in no danger of biohazard. Therefore, silkworm is excellent tool for drug screening and safety test.

I. Test systems

(1) Disease model therapeutic effect test

We previously constructed various disease silkworm models including bacterial or virus infection.

(2) Natural immunity stimulation test

Silkworm lacks acquired immunity but instead protects itself through innate immunity. We found that muscle of silkworm is contracted by stimulation of innate immunity.

(3) Safety (Toxicity and pathogenicity) test

Pathogenic bacteria can be detected by their silkworm killing activity. Since lethal doses per animal weight in silkworm are consistent with those in mammals, poison of about 1/100,000 of lethal dose in human can be detected.

(4) Drug kinetics test

Gastrointestinal absorbability of compounds can be examined by removed midgut.

II. Advantage of silkworm as model animal

(1) Low in cost

Silkworm is in low cost compared to mouse.

(2) Little conflict with ethical problem

It is harder than ever to use mammals for experiments because of kindness to animals.

(3) Rapid and convenient assay

We can obtain data rapidly and conveniently with small doses of samples.

(4) Injection into not only hemolymph but also midgut

Silkworm can be injected into not only hemolymph but also midgut. Each corresponds to intravenous injection or peroral administration. (Fig. 1).

(5) No danger of biohazard

Silkworm is in no danger of biohazard because it is inescapable from laboratory.

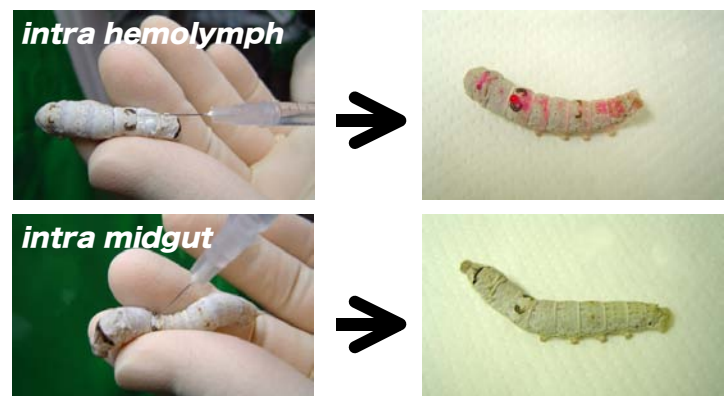


Fig. 1. Injection into hemolymph and midgut

When sting is shallow, red ink is injected into blood (Upper). On the other hands, when sting is deep, it is injected into midgut and silkworm is not stained (Bellow).

III. Principles and examples of test systems

(1) Disease model therapeutic effect test

A. Bacterial infection model

Silkworm is died after injection of pathogenic bacteria such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Vibrio cholera* and others but it is survived by injection of antibiotics including chloramphenicol (Kaito *et al.* 2002) (**Fig. 2**). Since 50% effective doses (ED₅₀) of antibiotics in silkworm are consistent with those in mammals (Hamamoto *et al.*, 2004) (**Table 1**), silkworm is useful for antibiotics screening.

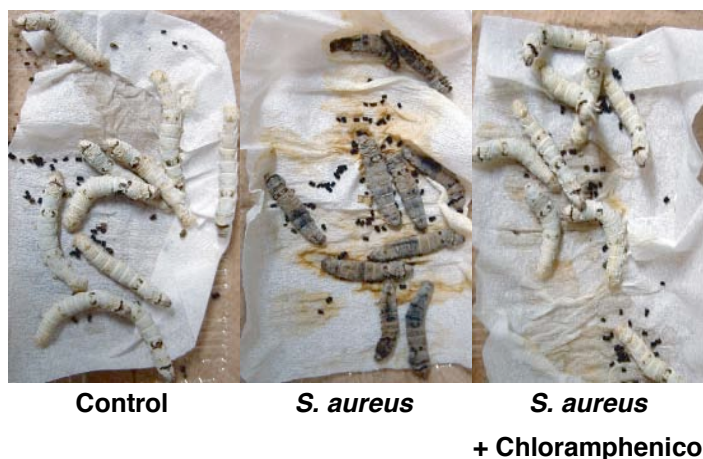


Fig. 2. Cure of bacterial infection by antibiotics in silkworm

Table 1. ED₅₀ of antibiotics in silkworm or mammals infected with *S. aureus*

Antibiotics	ED ₅₀ (mg/g•animal) ^a	
	Silkworm	Mouse
Teicoplanin	0.3	0.1
Vancomycin	0.3	1
Minocycline	4	1
Flomoxef	0.2	0.3
Linezolid	9	4

^a 50% effective dose per gram animal

Conventional method of antibiotics screening is that antibacterial activities are examined and the positive substances are further subject to therapeutic effect test but almost are unstable *in vivo* because of problem for ADME (administration, distribution, metabolism and excretion). On the other hands, our method is that therapeutic effects are first examined and antibacterial activities are confirmed afterwards. Thus, low cost, rapid and convenient silkworm system allows high efficient screening.

B. Fungi infection model

Silkworm is also died by fungi such as *Candida albicans*, *Candida tropicalis*, *Cryptococcus neoformans* and *Aspergillus fumigantes* and it is cured by antifungals including fluconazole.

C. Virus infection model

Nuclear polyhedrosis virus (baculovirus) kills silkworm and ganciclovir or foscarnet that is the antiviral agent used in the treatment of human herpesvirus or cytomegalovirus infection (Orihara *et al.*, 2008). Thus, we can search for antiviral agents using baculovirus as a model virus (**Example 1**).

Example 1: Purification of antiviral agents from herbal medicines

Using the infection model, the antiviral activity of herbal medicines was screened and it was found that the Japanese traditional medicine Mao-to had a therapeutic effect (**Fig. 1A**). Based upon the therapeutic activity, an antiviral substance, cinnzelanine, was purified (**Fig. 1B**).

It is actually the rare case that any active substances are purified on the basis of therapeutic effects of individual animals. The clear index of life or death, simple and rapid measurement of the activity and small doses of materials enough to be injected into silkworm allow the purification.

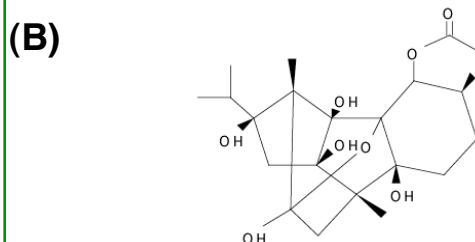
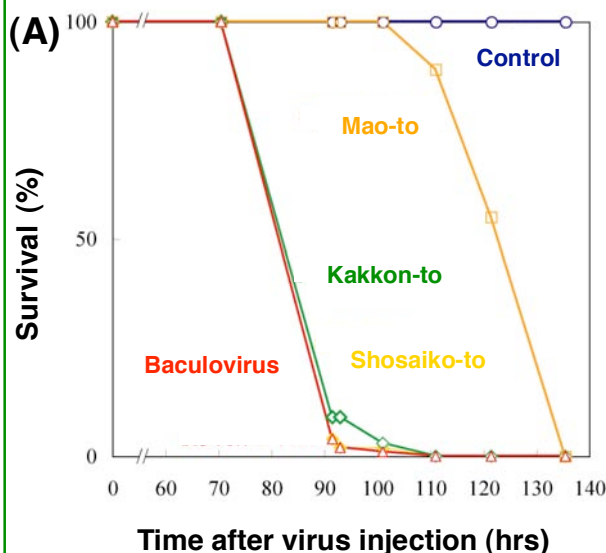


Fig. 1. Therapeutic activities of herbal medicines (A) and the purified antiviral agent (B)

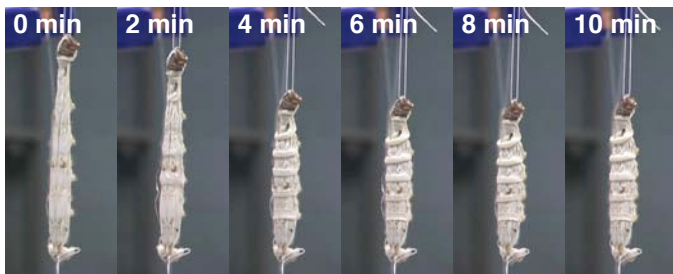
(2) Natural immunity stimulation test

Silkworm lacks acquired immunity but instead protects itself through innate immunity. We found that muscle of silkworm is contracted by stimulation of innate immunity (Ishii *et al.*, 2008) (Figs. 3 and 4). On the basis of the muscle contraction, we can search for immunostimulatory agents.

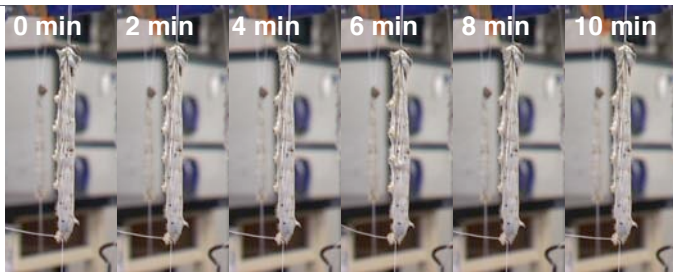
Since the muscle contraction does not respond to lipopolysaccharide (LPS), this assay is able to avoid false positive by the contaminated bacteria in the test samples as opposed to the conventional method of cytokine induction in mammal lymphocytes.

(A)

(i) Innate immunity stimulation factor



(ii) Saline



(B)

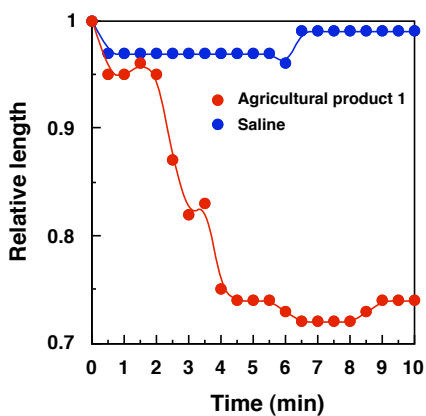


Fig. 3. Muscle contraction by stimulation of innate immunity in silkworm

Head and midgut of silkworm are removed and the muscle specimen is hung (A). Injection of innate immunity stimulation factor leads to muscle contraction within 10 minutes (A and B).

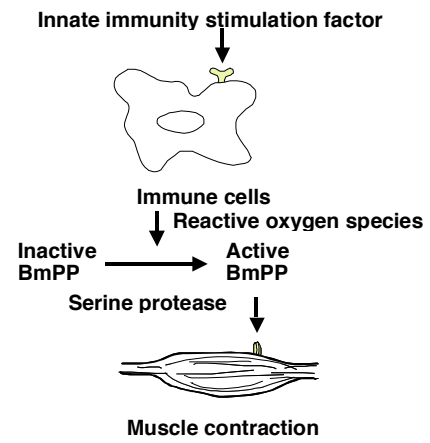


Fig. 4. A mechanism of muscle contraction by innate immunity stimulation.

Immune cells produce reactive oxygen species and serine protease activates paralytic peptide that paralyzes muscle.

(3) Safety test

A. Pathogenicity test

Since pathogenic bacteria such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Vibrio cholera* and others kill silkworm (**Fig. 2**, p2) but *Escherichia coli* laboratory strain does not, they can be detected by their killing activity.

Silkworm is died after injection of pathogenic bacteria within one or two day(s) and the bacteria that cause food poisoning and in-hospital infection could be immediately detected.

B. Toxicity test

Not only ED₅₀ of antibiotics (**Table 1**, p2) but also 50% lethal doses (LD₅₀) of poisons (**Table 2**) in silkworm are consistent with those in mammals (Hamamoto *et al.*, 2008). Taking into consideration that lethal doses per animal weight are comparable, about 1/100,000 of lethal dose in human could be detected.

Table 2. LD₅₀ of poisons in silkworm and mammals

Poison	LD ₅₀ (μg/g • animal) ^a	
	Silkworm	Mouse / Rat
Ethanol	9500	10000
Methanol	2100	2130
DMSO	33000	12000
DMF	16000	2800
Phenol	310 - 3100	310
<i>m</i> -cresol	0.63	2
NaCl	9100	4000
FuSO ₄	220	1500
CuSO ₄	310	960
Sodium azide	380	45
KCN	115	8.7

^a 50% lethal dose per gram animal

Physical and chemical methods are commonly used for the detection of poisons but it seems to be impossible that all of detecting tests could be applied to one sample. We propose safety test of agricultural products, foods and environments such as water and soil using silkworm as “coal mine canaries.”

(4) Drug kinetics test (Gastrointestinal absorbability test)

It is because drug kinetics in silkworm and mammals are similar to each other that ED₅₀ of antibiotics (**Table 1**, p2) and LD₅₀ of poisons (**Table 2**) are consistent between these animals; silkworm possess drug-metabolizing enzymes that are identical to those in mammals and their gastrointestinal absorbability of drug is also comparable to each other. In silkworm, vancomycin is low in gastrointestinal absorbability and it is not effective through peroral administration as well as the case in mammals (Hamamoto *et al.*, 2004) (**Table 3**, **Fig. 5**).

Table 3. Effect of difference in the administration method on ED₅₀ of antibiotics in silkworm

Antibiotics	ED ₅₀ (μg/g • animal) ^a		
	<i>i. h.</i> ^b	<i>i. m.</i> ^c	Peroral
Chloramphenicol	9	11	40
Tetracycline	0.4	1	8
Vancomycin	0.3	> 700	> 400
Kanamycin	3	> 700	> 500

^a 50% effective dose per gram animal

^b *intra hemolymph*

^c *intra midgut*

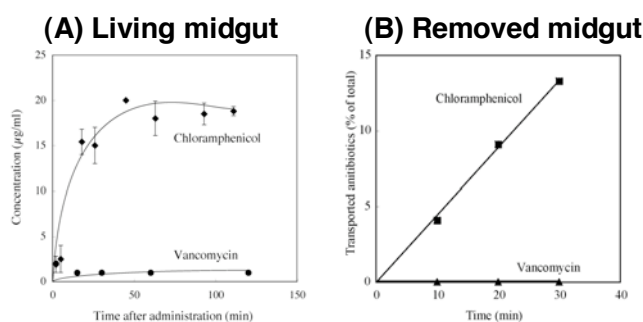


Fig. 5. Gastrointestinal absorbability of antibiotics in living (A) or removed (B) midgut of silkworm

Gastrointestinal absorbability test can be carried out *in vitro* using removed midgut of silkworm (**Fig. 6**).



Fig. 6. Gastrointestinal absorbability test *in vitro* using removed midgut of silkworm

IV. Publication

1. Hamamoto, H. *et al.* (2008) *CBP*, in press.
2. Ishii, K., Hamamoto, H., Kamimura, M. and Sekimizu, K. (2008) Activation of the silkworm cytokine by bacterial and fungal cell wall components via a reactive oxygen species-triggered mechanism. *J. Biol. Chem.*, **283**, 2185-2191.
3. Orihara, Y., Hamamoto, H., Kasuga, H., Shimada, T., Kawaguchi, Y. and Sekimizu, K. (2008) A silkworm-baculovirus model for assessing the therapeutic effects of anti-viral compounds: characterization and application to the isolation of anti-virals from traditional medicines. *J. Gen. Virol.*, **89**, 188-194.
4. Hamamoto, H., Kamura, K., Razanajatovo, I. M., Murakami, K., Santa, T. and Sekimizu, K. (2005) Effects of molecular mass and hydrophobicity on transport rates through non-specific pathways of the silkworm larva midgut. *Int. J. Antimicrob. Agents* **26**, 38-42.
5. Hamamoto, H. and Sekimizu, K. (2005) Evaluation of the therapeutic effects of antibiotics using silkworm as an animal model. *Res. Adv. Antimicrob. Agents Chemother*, **5**, 1-23.
6. Hamamoto, H., Kurokawa, K., Kaito, C., Kamura, K., Manitra Razanajatovo, I., Kusuhara, H., Santa, T. and Sekimizu, K. (2004) Quantitative evaluation of the therapeutic effects of antibiotics using silkworms infected with human pathogenic microorganisms. *Antimicrob. Agents Chemother*, **48**, 774-779.
7. Kaito, C., Akimitsu, N., Watanabe, H. and Sekimizu, K. (2002) Silkworm larvae as an animal model of bacterial infection pathogenic to humans. *Microb. Pathog.*, **32**, 183-190.

V. Patent application

1. Genome Pharmaceuticals Institute Co., Ltd., Japan Patent Application No. 2008-063817, April 7, 2008 (**Pathogenicity test**).
2. Genome Pharmaceuticals Institute Co., Ltd., Japan Patent Application No. 2007-214006, August 20, 2007 (**Toxicity and drug kinetics tests**).
3. Genome Pharmaceuticals Institute Co., Ltd., the University of Tokyo and Imagine Global Care Corporation, Japan Patent Application No. 2007-102918, April 10, 2007 (**Natural immunity stimulation test**).
4. Genome Pharmaceuticals Institute Co., Ltd., Japan Patent Application No. 2004-155989 (2006-513824), May 26, 2004 (PCT/JP2005/07382) (**Virus infection model**).
5. Genome Pharmaceuticals Institute Co., Ltd., Japan Patent Application No. 2000-177565 (2001-583180), May 11, 2000 (**Bacterial and fungi infection models**).

Genome Pharmaceuticals Institute

Genome Pharmaceuticals Institute Co. Ltd. is the bio-venture company of cooperation between industry and academia that is established to rely on Prof. Sekimizu's research to put into practical use. We aim to develop novel drugs using silkworm as experimental animal. We previously constructed various disease silkworm models including bacterial or virus infection, diabetes and so on.

We recently search for antibiotics using bacterial infection model (This work is supported by funds from National Institute of Biomedical Innovation of Japan (NIBIO)) and develop "evidence-based" functional foods or supplements using silkworm, especially that activate natural immunity using muscle contraction assay. Moreover, we propose safety test of agricultural products, foods and environments using silkworm as "coal mine canaries" (This work is supported by funds from Japan Science and Technology Agency (JST)).

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