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Editorial Board Members

CONTENTS

Volume 10, Number 1, 2016

Editorial

Reviews

CONTENTS (*Continued*)

Original Articles

Guide for Authors

Copyright

Editorial

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Using silkworms as a laboratory animal to evaluate medicines and foods

Kazuhisa Sekimizu*, Hiroshi Hamamoto

Laboratory of Microbiology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan.

Summary **For this special issue, we, the Editors of Drug Discoveries & Therapeutics, have asked researchers who are using silkworms to actively develop drugs and study foods to summarize their recent work. Our profound hope is that this special issue encourages researchers who are helping to develop the new field of "using silkworms as a laboratory animal to evaluate medicines and foods".**

Keywords: Silkworm, *Bombyx mori,* animal model, antibiotics, drug discovery

Many countries are facing an aged population unlike that ever seen before. People fervently want better health and a longer lifespan. Therefore, medical care systems need to be developed in various fields. An important task for both medical and economic experts is to develop new medicines to treat various diseases. People also want evidence that foods can keep them healthy. The use of laboratory animals is a key issue in these situations involving the production of medicines and foods. Mammals like mice and rats have been used as laboratory animals, but sacrificing a large number of mammals poses a serious problem in terms of cost as well as in terms of animal welfare. A recent highlighting on animal welfare is seriously hampering the development of medicines in Europe, and countries in Asia will need to address this problem in the near future.

The use of invertebrates is a possible solution to the problems of high costs and ethical issues caused by the use of mammals. Studies have proposed that fruit flies (*Drosophila melanogaster*) and nematodes (*Caenorhabditis elegans*) could serve as laboratory animals in order to evaluate the therapeutic effects of drug candidates (*1,2*). A problem with the use of these invertebrates is that they are too small, making injection of precise volume of sample difficult. Techniques to inject animals as models need to be established in order to evaluate drug candidates.

Silkworms are the larvae of the silk moth, *Bombyx mori*. Methods of rearing silkworms have been established over a history of sericulture spanning more than five thousand years (*3*). Here, we would like to propose a new concept: "use of silkworms for drug development". Silkworms are akin to a silk-producing factory. Over the past few years, silkworms have been used to produce recombinant proteins (*4*). However, little attention has been paid to the usefulness of silkworms as laboratory animals in the field of medicine and pharmaceutical sciences. Therefore, "use of silkworms in drug development" is a novel idea substantiated by a long history of sericulture. Sericulture is still expanding in Asia. Hopefully, the concept of "using silkworms in drug development" will develop as a new industry.

Silkworms are useful at evaluating drug candidates (*5-8*) as well as foods (*9*). People are looking to food for nutrition and to supplements for substances that provide better health. Silkworms are particularly useful at evaluating food that may contain substances that activate innate immunity. Determining whether or not immunity is activated in humans and other mammals is usually difficult. We found that stimulating innate immunity in silkworms causes muscle contractions (*10,11*). Activation of cells responsible for innate immunity in silkworms leads to enhancement of immune cells that results in the activation of a cytokine that has the pharmacologic capacity to induce muscle contractions. We can easily use this phenomenon to screen food for substances that activate immunity. Beta-glucans in yeast cells and peptidoglycans in lactic acid bacteria have potent activity according to a silkworm muscle contraction assay (*10*).

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Substances that cause innate activation were previously evaluated based on cytokine production by macrophages isolated from mammals, but lipopolysaccharides (LPS) derived from the outer structure of Gram-negative bacteria like *Escherichia coli* produce false positives at very low concentrations. The clear advantage of the silkworm muscle contraction assay is that it does not respond to LPS. Since we can easily develop models of various conditions like infections, diabetes, and cancer, silkworms should prove highly useful at evaluating the various functions of food components.

For this special issue, we, the Editors of Drug Discoveries & Therapeutics, have asked experts, who are using silkworms to actively develop drugs and study foods to summarize their recent results. Our profound hope is that this special issue encourages researchers who are helping to develop the new field of "using silkworms as a laboratory animal to evaluate medicines and foods".

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Review

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Can the silkworm (*Bombyx mori*) be used as a human disease model?

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Summary ***Bombyx mori* (silkworm) is the most famous lepidopteran in Japan. *B. mori* has long been used in the silk industry and also as a model insect for agricultural research. In recent years, *B. mori* has attracted interest in its potential for use in pathological analysis of model animals. For example, the human macular carotenoid transporter was discovered using information of *B. mori* carotenoid transporter derived from yellow-cocoon strain. The *B. mori* carotenoid transport system is useful in human studies. To develop a human disease model, we characterized the human homologs of *B. mori*, and by constructing KAIKO functional annotation pipeline, and to analyze gene expression profile of a unique *B. mori* mutant strain using microarray analysis. As a result, we identified a novel molecular network involved in Parkinson's disease. Here we describe the potential use of a spontaneous mutant silkworm strain as a human disease model. We also summarize recent progress in the application of genomic information for annotation of human homologs in *B. mori*. The *B. mori* mutant will provide a clue to pathological mechanisms, and the findings will be helpful for the development of therapies and for medical drug discovery.**

Keywords: Silkworm, human disease model, *Bombyx mori* mutant, Parkinson's disease, translucent larval skin

1. Introduction

The silkworm (*Bombyx mori*), which produces silk fiber in its silk glands, is the most famous lepidopteran in Japan. It was domesticated by humans more than 5,000 years ago in China. *B. mori* cannot survive without human help.

In the 19th century, silkworm was an important livestock animal in Japan. Nature and Science Museum of Tokyo University of Agriculture and Technology (*http://www.tuat.ac.jp/~museum/*) displays several ukiyo-e that depicts scenes of sericulture at that time. One ukiyo-e depicts women reeling cocoons (Figure 1A), and another depicts women rearing silkworms (Figure 1B). Thus, rearing silkworms, reeling cocoons and weaving cloth were done by the women. These methods of sericulture were recorded in some books. Silkworms were important possessions for the Japanese people. They preferred colored to white cocoons, and variety of colored cocoons were produced by Japanese sericultureists till the 19th century. However, it has not been clear why whited cocoon strains were standardized at the early 20th century. The Japanese people collected many silkworm strains from the Europe and China. Because Japanese sericultureists were seeking for silkworm strains which were disease-resistant and made the big cocoon. In this process, Japanese sericultureists obtained various phenotypes, and discovered many unique mutants. Therefore, Japanese sericultureists have maintained these many silkworm races of these for the future gene resource need.

Additionally, Japanese geneticist's Toyama rediscovered the Mendel's law of heredity in animal using white and yellow cocoon race of *B. mori* in 1906 (*1*). Thereby, the phenotypes of *B. mori* were genetically linked to the causative genes on the linkage maps of *B. mori*. Currently, 456 mutant strains are maintained at the National Bio Resource Project (NBRP) KAIKO (*http://*

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Figure 1. Sericulture and silkworm depiction on Ukiyoe.

silkworm.nbrp.jp/) located at Kyushu University.

The silkworm has been used as a model insect for agricultural research for several reasons: *i*) the majority of agricultural pests are lepidopterans, *ii*) its genome sequence is well characterized, *iii*) various genetic mutants are available, and *iv*) silkworm is amenable to transgenic, knock-out, and microarray technologies (*2-7*). However, to date, silkworms have never been used as human disease models.

Here we describe the potential use of a spontaneous silkworm mutant strain as a human disease model, based on our current study (*8*). We also summarize recent progress in the application of genomic information for the annotation of human homologs in *B. mori*. These findings will help elucidate molecular mechanisms of human diseases.

2. The human macular carotenoid transporter discovered using an information of *B. mori*

There are several known colored cocoons: yellow, pink, golden yellow, straw, green, and sasa (yellowish green). Yellow-, pink-, golden-yellow-, and flesh-colored cocoon pigments are derivatives of carotenoids (*9*), whereas sasa- and green-cocoon pigments are derivatives of flavonoids (*10,11*). These pigments protect pupae from sunlight as antioxidants (*12*). A carotenoid-binding protein (CBP) was identified in a *B. mori* yellow-cocoon strain in 2002 (*13*) as an intracellular carotenoid transporter in the silk gland by a biochemical approach. Interestingly, CBP was categorized as the first member of a steroidogenic acute regulatory protein (StAR) family whose members bind only carotenoids in their lipid-binding domains.

Landrum *et al*. (*14*) have reported that carotenoids are accumulated in the human macula as macular pigment. A low concentration of macular pigment increases the risk of age-related macular degeneration from blue light damage. However, sustained intake of dietary carotenoids may reduce the risk of age-related human macular degeneration (*14*). Thus, identification of a macular carotenoid transporter is important for elucidation of the molecular mechanism of macular degeneration.

The antibody to *B. mori* CBP has cross-reactivity to macular carotenoid transporter identified from human retina and layers of the primate macula where the macular carotenoid pigment is at its highest concentration (*15*). The human macular carotenoid transporter StARD3 (also known as MLN64) was discovered by Bernstein *et al*. in 2009 (*16*).

The *B. mori* CBP information and antibody were helpful for identification of the human macular carotenoid transporter. Therefore, the common molecular mechanisms were present in the carotenoid transport system between *B. mori* and human.

In the present study, a *B. mori* cocoon-color mutant was helpful for elucidation of the molecular mechanism of age-related human macular degeneration (*17*).

3. How many human orthologs does silkworm have?

A draft silkworm genome sequence was completed by Chinese and Japanese groups in 2004 (*2,3*). In 2013, Suetsugu *et al*. (*4*) reported that the silkworm genome contained 16,823 gene loci, based on sequence analysis of cDNA data set. However, these data are not included in public databases currently. We accordingly identified human homologs using the Ensembl Metazoa (*18*) predicted-protein data set of *B. mori*.

There are challenges in using silkworm genome information. Silkworm genome sequences have been analyzed, but the functional annotation of the genes still remains obscure. If we can annotate the *B. mori* genome in the same manner as the *Drosophila melanogaster* genome, we can use the public databases to mammalian model organisms and commercial pathway softwares for microarray analysis or next-generation sequencing (NGS) data. Finally, we can analyze these big data from microarray or NGS analysis as deeply as for mammalian model organisms. *D. melanogaster* has been used as a human disease model in studies involving gene-gene interactions. A systematic BLAST search (*19*) revealed 548 human disease-associated genes in *D. melanogaster*. Importantly, *D. melanogaster* is the only insect species in which gene annotations have been extensively assigned (*20,21*), and many human disease models have been developed. However, the analysis of human disease-associated genes was reported by Reiter *et al*. (*19*) in 2001 and the complete human genome was not available at that time. Thus, we needed the re-analysis of updated human gene set in the current study. To annotate silkworm genome information, we identified human homologs common to *B. mori* and *D. melanogaster*; we obtained the cDNA sequence sets of these species from Ensembl Metazoa, and we performed systematic BLAST search to identify human homologs in these species. *B. mori* contained 8,469

Table 1. Comparison of human homologs between *Bombyx mori* and *Drosophila melanogaster*

Species	Human homolog transcripts	Total transcripts	Human homolog genes	Total (genes)	Ratio (%) of human homolog/total genes
B. mori	8469	14623	8469	14623	58
D. melanogaster	21230	30362	8815	13918	63

Table 2. Gene enrichment analysis of human homolog genes in *Bombyx mori*

KEGG ID	KEGG pathway	Count	*p* value
hsa05016	Huntington's disease	82	2.1E-4
hsa05010	Alzheimer's disease	68	1.1E-2
hsa05012	Parkinson's disease	51	1.1E-2
hsa04120	Ubiquitin mediated proteolysis	67	7.0E-5
hsa00190	Oxidative phosphorylation	54	2.5E-2
hsa03050	Proteasome	31	8.7E-6

4,020 genes shared with 57 Kyoto Encyclopedia of Genes and Genomes pathways.

and *D. melanogaster* contained 8,815 human homologs (Table 1). Thus, *B. mori* had 58% of the human homologs in the genes. Furthermore, we characterized these human homologs in *B. mori* by enrichment analysis using the DAVID bioinformatics database (*https://david.ncifcrf.gov/home.jsp*). The human homologs were included in 57 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and the genes in the most conserved pathways in the DAVID analysis were concerned in neurodegenerative disease, oxidative stress, and protein degradation-associated genes (Table 2). In addition, these pathways were also conserved in *D. melanogaster*, and corresponding related human disease models were developed. The FlyBase Human Disease Model Report List (*http://flybase.org/static_pages/FBhh/browse.html*) contains 59 human disease models in *D. melanogaster*. The basic information of human disease related genes were obtained from Online Mendelian Inheritance in Man database. Human disease related fly ortholog was modified as functional disturbance, and the genetically modified fly was created. These human disease model flies are reported in the public database and stock centers. Accordingly, *B. mori* may have potential use as a human disease model similar to *D. melanogaster*.

4. Abundance of spontaneous silkworm mutant stocks in Japan

The NBRP KAIKO also maintains 456 spontaneous mutant *B. mori* strains. These genes responsible for each mutation have been mapped in the *B. mori* linkage map information. User can choose the mutant which correspond to gene symbols and mutant strains on the NBRP web site (*http://www.shigen.nig.ac.jp/silkwormbase/ViewCausativeGene.do?*). Some causative genes of the product have been discovered and are shown in a table on the NBRP web site. However, many causative genes in *B. mori* mutants have not been identified. Importantly, spontaneous mutants have unique phenotypes that do not appear in other model species. For instance, there are a variety of cocoon colors, egg colors, and larval skin colors and patterns; translucent larval skin; and black-colored adults. If we can identify the causal genes responsible for these phenotypes as human homolog, the discovery will contribute to identify novel molecular mechanisms that could not be detected using other model organisms.

Thus, *B. mori* has many valuable spontaneous mutants in Japan. We propose that *B. mori* is a good human disease model candidate, on the basis of these spontaneous mutants.

5. KAIKO functional annotation pipeline is useful for screening target molecules

B. mori has 26 mutants in uric acid metabolism. The common phenotype of these mutants is translucent larval skin (*22*). Eight genes have been identified as causative genes in translucent larval skin mutants (*23-31*). These genes are involved in the synthesis or uptake of uric acid.

To analyze the *B. mori* translucent larval skin mutant strain o751 (*op*), we constructed KAIKO functional annotation pipeline using corresponding information of *B. mori* human homologs and human genes (Figure 2).

The *B. mori op* mutant is classed as a translucent larval skin mutant (Japanese name, aburako) and displays occasional unique actions such as vibration. Classical linkage analysis has shown that the *op* gene is located on chromosome 23, and involved in the phenotypes of the extraordinarily high mortality, particularly in the pupal stage, and the male infertility except for the oily mutation (NBRP silkworm database; *http://www.shigen.nig.ac.jp/silkwormbase/ViewStrainDetail.do?id=309*). We investigated gene expression in the *B. mori op* mutant using microarray analysis with KAIKO functional annotation pipeline. We identified a novel uric acid synthesis-modulated pathway (Figure 2, gray-colored molecule) (*8*).

6. Parkinson's disease and uric acid

Parkinson's disease (PD) remains an incurable disease. Its mechanisms responsible for dopaminergic neuronal cell degradation cause oxidative stress or protein accumulation by ubiquitin proteasome failure, and this damage depletes dopamine levels in substantia

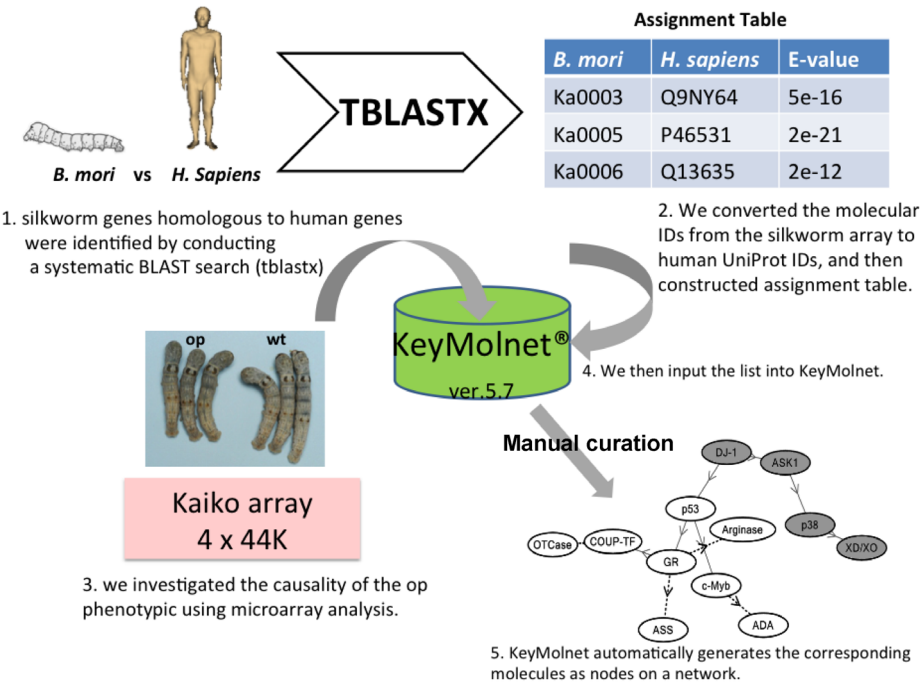

Figure 2. Screening of target molecule using KAIKO functional annotation pipeline.

nigra neurons (*32,33*). Genetic studies have identified 21 genes associated with PD at different loci based on family linkage analysis [PD; Online Mendelian Inheritance in Man 168600]. PD-associated gene knockout animal models have been developed as familial PD models (*34*).

The majority of sporadic PD onset is caused by environmental stress (*35,36*), and a molecular mechanism of oxidative stress has been developed. In animal models of sporadic PD, oxidative stress has been simulated using mitochondrial complex I inhibitors (*37*).

The final product of purine metabolism, uric acid, plays an important role as a physiological antioxidant (*38*). Several groups have reported a correlation between decreased plasma uric acid concentrations and clinical progression and stage of PD (*39-45*). Conversely, high plasma uric acid concentrations in hyperuricemia may reduce the risk and delay the progression of PD, but it increases the risk of cardiac diseases (*46*). Thus, uric acid has a dual function in organisms. In the case of PD, uric acid may be expended to resist oxidative injury (*47*); however, the molecular mechanism underlying the decrease in plasma uric acid concentration in advanced clinical stages of PD has not been analyzed using either of these model animals. Owing to the lack of adequate animal models, the function of uric acid in PD pathogenesis is poorly understood.

7. Why is a silkworm model a good candidate for the analysis of Parkinson's disease and uric acid?

It is well established that the uric acid metabolism process uses *B. mori* mutants. Uric acid is synthesized mainly in the fat body and is thereafter transported to the integument via the hemolymph. It is the end product of purine degradation *via* xanthine/hypoxanthine reactions catalyzed by xanthine dehydrogenase. It is eliminated through the Malpighian tubules. Uric acid accumulates as urate granules and produces a whitening of the integument. In translucent larval skin mutants, it shows abnormal accumulation in the integument (*22*).

Uric acid plays a protective role against photooxidative stress in *B. mori*, as shown by a markedly reduced survival rate in larvae under UV irradiation with injection of allopurinol, an inhibitor of uric acid synthesis (*48*). It directly scavenges oxygen radicals and may play an important role in protection against environmental oxidative stress in *B. mori*.

Only *B. mori* translucent larval skin mutants show abnormality in integument color in the larval developmental stage. Other model organisms do not show a similar phenotype.

In human studies, plasma uric acid concentrations decrease following the clinical progression and stage development of PD (*39-45*). However, molecular mechanisms underlying reduction in plasma uric acid concentrations remain unknown. Reduced plasma uric acid concentrations are due to consumption of uric acid as an antioxidant in PD. Furthermore, the causative gene of human PD induces strong oxidative stress in the central nervous system (*47*). Moreover, the regulation of xanthine dehydrogenase phosphorylation in the uric acid synthesis pathway is unclear.

We accordingly investigated gene expression in the *B. mori op* mutant using KAIKO functional annotation pipeline for analysis of microarray data. We identified

a novel uric acid synthesis-modulating pathway (Figure 2). We speculated that these molecules relate to the phosphorylation of the protein (*8*). However, we were unable to identify the *op* causative gene in the present study.

Molecular mechanisms associating decreased plasma uric acid concentrations with PD remain obscure. *B. mori* translucent larval skin mutants provide promising clues for elucidation of these mechanisms and for development of therapies and drugs for PD. Further study of genes with common function in the uric acid synthesis pathways of humans and *B. mori* is warranted.

Do you now believe that silkworms can be used as human disease models?

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Review

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Usefulness of silkworm as a host animal for understanding pathogenicity of *Cryptococcus neoformans*

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Summary **We propose *Cryptococcus neoformans* infection model using silkworm for understanding cryptococcosis and screening of therapeutically effective antibiotics. Silkworm is an insect whose rearing methods were established through a long history of the sericulture industry. Silkworm facilitates experiments using a large number of individuals because of low cost for rearing and few ethical problems caused by killing animals. Silkworm can be reared at 37˚C to perform infection experiments at same temperature to human body. Injection of accurate amounts of samples into hemolymph of silkworm by usual syringes is easy to be done since silkworm has an appropriate size to handle. Moreover two injection methods, injection into hemolymph and intestine, are distinguishable for silkworms. The former is correspondent to intravenous injection, and the latter is to oral administration in humans. Taking these advantages of silkworms as host animals, it is possible to evaluate the virulence factors in *C. neoformans* and the therapeutic efficacy of antifungal agents.**

Keywords: *Cryptococcus neoformans*, infectious disease, silkworm

1. Introduction

Cryptococcus neoformans is a pathogenic fungus that causes cryptococcosis in humans (*1*). *C. neoformans* is frequently isolated from immunocompromised patients. Cryptococcosis is one of the most causes of death in AIDS patients (*2*). Basic study using animal models that imitates human infectious disease is necessary to understand pathogenicity of *C. neoformans* and to establish prevention and therapeutic strategies against cryptococcosis. Various *C. neoformans* infection models with mammalian hosts have been proposed (*3-5*). Mammalian models, however, have problems of not only high cost but also of ethical issues from a view of animal welfare. Therefore, establishment of invertebrate animals for searching virulence factors of *C. neoformans* and for screening of therapeutic agents is desired. Invertebrate animals have advantages compared to mammals: *1)* low cost for rearing, *2)* smaller space needed for rearing, *3)* less ethical issues by killing animals, and *4)* less amount of samples because of smaller body size (Table 1). At present, besides silkworm (*Bombyx mori*) proposed by us (*6*), fruit fly (*Drosophila melanogaster*), nematode (*Caenorhabditis elegans*), and larvae of greater wax moth (*Galleria mellonella*) are proposed as host animals of *C. neoformans* infection (*7-9*). In this review, we describe usefulness of these invertebrates as host of *C. neoformans* infection.

2. *C. neoformans* infection model using silkworm

Silkworm is a larva of domesticated silkmoth, *Bombyx mori*. The rearing method is well-established during a long history of sericulture in Asian countries. We previously proposed various disease models, such as infectious diseases by pathogens, diabetes, and drug-induced tissue injury, and use of these systems for screening of drug candidates (*10-13*). Among them, infection models including fungal infection are highly effective for screening virulence factors of pathogens and therapeutically effective antibiotics (*14,15*). Silkworm fungal infection models were reported for four species of fungi, *Cryptococcus neoformans*, *Candida albicans*, *Candida grabrata*, and *Candida tropicalis* (*16*). Several

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Table 1. *In vivo* infection models of *Cryptococcus neoformans*

Items	Model animals	Cost for rearing	Space for rearing	Application to the ethics committee	Escape ability to require the biosafety	Time required for die after injection	Temperature after infection	Quantitative injection of samples by a syringe	Reported route of administration	Individual weight
Silkworm	*Bombyx mori*	Low	Small	Not necessary	Low	2-3 days	37°C	Easy	Intrahemolymph, intragut injections	1-2 g
Fruit fly	*Drosophila melanogaster*	Low	Small	Not necessary	High	3-4 days	25°C	Difficult	Intrahemolymph injection, oral administration	0.5-2 mg
Nematode	*Caenorhabditis elegans*	Low	Small	Not necessary	Low	2-25 days	25°C	Difficult	Oral administration	1 μg
Larvae of greater wax moth	*Galleria mellonea*	Low	Small	Not necessary	Low	4-20 days	30°C, 37°C	Easy	Intrahemolymph injection	250 mg
Mice	*Mus musculus*	High	Large	Necessary	High	6-40 days	37°C (body temperature)	Easy	Intratracheal, intravenous, intraperitoneal injection	15-40 g

ND: Note Determined.

fungal virulence factors were identified using these fungal models (*17,18*). We previously reported that silkworm *C. neoformans* infection model was useful for quantitative evaluation of *C. neoformans* pathogenecity and therapeutic effects of antifungal drugs (*6*). Silkworm survive at least three days at 37˚C, therefore, silkworm can be used for infection experiments at 37˚C (*19*). Injection of live fungal suspension of *C. neoformans* H99 strain into silkworm hemolymph causes killing effects at 37˚C. *C. neoformans* Serotype A, which has high pathogenicity against mammals, was shown to kill silkworm with less number of fungi than Serotype D, which has low pathogenicity against mammals. In other words, silkworm infection model can distinguish strains having different levels of pathogenicities.

Deletion mutants of *gpa1*, *pka1*, and *cna1* genes, which were reported to be needed to exhibit pathogenicity of *C. neoformans* against mammals (*20-22*), also showed higher LD_{50} values than that of parent strain. This means that these genes are also needed to exhibit pathogenicity against silkworms as well as mammals. Intra-hemolymph injections of amphotericin B, flucytosine, fluconazole, and ketoconazole showed therapeutic effect against death of silkworm by *C. neoformans* infection. On the other hand, amphotericin B, which is not absorbed from gut in mammals, did not show the therapeutic effect by intra-gut injection, which corresponds to oral administration in humans. This result can be explained by that amphotericin B is not absorbed from gut also in silkworm. From these results, we expect that silkworm *C. neoformans* infection model is useful as an alternative method to evaluate therapeutic efficacy of antifungal drugs.

3. *C. neoformans* infection models using other invertebrate models

D. melanogaster, a fruit fly, is widely used as a model animal (*23*). An advantage of *D. melanogaster* as an experimental animal is that different kinds of genetic approaches can be applicable (*24*). Using mutant libraries of *D. melanogaster*, the host immune system related to *C. neoformans* infection has been elucidated. In particular, mutants of Imd and Toll pathways, which are signal pathway related to innate immune system of *D. melanogaster*, were analyzed in the *C. neoformans* infection model (*8*). A mutant of Toll pathway was susceptible to *C. neoformans* infection, whereas a mutant of Imd pathway was not susceptible. Thus, Toll pathway plays a key role in the innate immunity against *C. neoformans*. Adult flies, not larvae, are generally used in infection experiments using *D. melanogaster*. Special micro injectors with glass syringes, not usual clinical syringe, are needed for injection, because the size of adults fly is very small, 2-3 mm. Therefore, determination of LD_{50} and ED_{50} by injection of precise volume of sample solution is very difficult. Experiments

with adults of *D. melanogaster* at 37˚C are not possible, since the flies cannot be reared at high temperatures.

C. elegans also provides excellent animal model to perform genetic studies (*25*). Genes related to innate immunity in *C. neoformans* infection were identified using *C. elegans* (*26-28*). Capsule and other virulence factors of *C. neoformans*, which are needed to exhibit the pathogenicity against mammals, were reported to be needed to exhibit the pathogenicity against *C. elegans* (*7*). *C. elegans* was also used to screen virulence factors of *C. neoformans* (*29-36*). Moreover, *C. neoformans* infection model with *C. elegans* was also used to evaluate therapeutic effects of antifungal reagents (*37,38*).

G. mellonella is large moth which belong to Lepidoptera, same as silkworm. *G. mellonella* has been studied as infection models of fungi including *C. neoformans* (*39-42*). *G. mellonella* is possible to perform infection experiments at 37˚C. Novel virulence factors of *C. neoformans* was also screened using the *G. mellonella* model (*43*). Evaluation of therapeutic effects of antifungal drugs was reported with an infection model of *C.neoformans* with *G. mellonella* (*9,44*). Since its big body size, *G. mellonella* has a capacity to collect a large volume of hemolymph similar to silkworm (*45*).

4. Conclusions

Invertebrate animal hosts, silkworm (*B. mori*), fruit fly (*D. melanogaster*), nematode (*C. elegans*), and larva of greater worm moth (*G. mellonella*), are expected to solve problems of high cost and ethical issues from a view of animal welfare in *C. neoformans* infection models using mammals, such as mice and rats. Silkworm has several advantages compared to *D. melanogaster* and *C. elegans*: *1)* bigger body size of individuals and lower motility, which facilitate quantitative injection of samples, *2)* survival at 37˚C, body temperature of human, and *3)* available for two types of injection ways, intra-hemolymph and intra-gut. Whereas, *D. melanogaster* and *C. elegans* have advantages of experimental systems for genetics, such transgenic techniques can be applicable for silkworms (*46-48*). Using these techniques, understanding of host immune system in silkworm responding to *C. neoformans* infection is important issue in future.

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Review

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A hyperglycemic silkworm model for evaluating hypoglycemic activity of Rehmanniae Radix, an herbal medicine

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Summary **Silkworm shows hyperglycemia after intake of diet containing large amount of glucose. The hyperglycemic silkworm model is useful for evaluation of anti-diabetic drugs. A hot water extract of Rehmanniae Radix, an herbal medicine, showed hypoglycemic effect against the hyperglycemic silkworms. This method is applicable for quick and simple evaluation of the hypoglycemic activities of different batches of Rehmanniae Radix. Our findings suggest that silkworms have a lot of merit as experimental animals for evaluation of various herbal medicines.**

Keywords: Hyperglycemia, silkworm, *in vivo* evaluation system, Rehmanniae Radix, quality check

1. Introduction

Crude extracts of herbal medicines are clinically used without extensive purification of active compounds. Herbal medicines are widely used in Asia. They are also used as a folk medicine in Europe and in America. Therapeutic activity of herbal medicines is known to be greatly affected by their origins and storage conditions, since the amounts of active compounds in herbal medicines are altered by those factors. Therefore, to ensure the maintenance of high quality of herbal medicines, establishment of methods for quality check is needed (*1*). In general, the quality check of herbal medicine is performed by analytical methods: liquid chromatography-mass spectrometry (LC-MS), fingerprint, quantitative analysis of multi-components by single-marker (QAMS), and thin layer chromatography bio-autographic assay (TLC-BAA) (*2*). These methods are aimed to identify species and content of secondary metabolites in herbal medicine. However, since active compounds in the herbal medicines are not identified in most cases, there are problems to ensure the qualities of the herbal medicines. To overcome the problem, establishment of novel quality check methods for monitoring therapeutic activity of the herbal medicines is desired. Previously mammalian animals have been used as model animals for evaluation of therapeutic effect of herbal medicine. However, the use of large numbers of mammalian animals causes serious problems, because it is costly and occurring the ethical issues in terms of animal welfare.

2. Usefulness of silkworms for evaluation of therapeutic effects of herbal medicines

We have proposed that silkworms are useful for evaluation of therapeutic effects and toxicities of drug candidates (*3-6*). We previously reported that therapeutic effects and pharmacokinetics of chemicals, such as anti-bacterial, anti-fungal, anti-viral drugs, in silkworm were similar to that in mammals (*3,4,7-10*). Silkworm can be easily handled for evaluation of therapeutic activities of drug candidates using large numbers of individual animals with low costs. Moreover, space needed for rearing silkworms is much smaller than that for mammals. We developed silkworm infection models to understand the molecular mechanisms for pathogenicity of bacteria and fungi (*3,10,11*). A number of virulence genes in *Staphylococcus aureus* could be identified by large-scale screening (*12*). The system could be utilized for functional analyses of the virulence genes of *Staphylococcus aureus* (*13-17*). Accurate volumes of sample solution can be easily administrated into hemolymph of silkworms by injection using a syringe. Such injection experiments are difficult to perform

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with other small invertebrates such as nematodes (*Caenorhabditis elegans*) and fruit flies (*Drosophila melanogaster*). Moreover, biochemical analyses are possible with silkworms, since relatively large amounts of hemolymph can be performed from silkworms. As mentioned above, silkworms have a number of advantages as an experimental animal for the evaluation of therapeutic activity of drug candidates including herbal medicines.

3. Evaluation of the hypoglycemic activities in extracts of Rehmanniae Radix using hyperglycemic silkworms

We previously reported that feeding of a high glucose diet causes hyperglycemia in silkworms (*18*). The hyperglycemic silkworm model is useful for evaluating the hypoglycemic effect of human insulin (*18*). We also demonstrated that some of the drugs clinically used for diabetes patients showed the hyperglycemic activities in the system. Rehmanniae Radix (RehR), a root of *Rehmannia glutinosa* Liboschitz var. *purpurea* Makino or *Rehmannia glutinosa* Liboschitz (Scrophulariaceae), is known to have hypoglycemic activity against mammals, and is widely prescribed for patients with diabetes (*19,20*). We asked whether the hypoglycemic effect of the RehR could be observed in the hyperglycemic silkworms. Since hot water extract of RehR abundantly contains a large number of monosaccharide such as glucose, sample administration of the hot water extract was expected to cause hyperglycemia in silkworm. Therefore, we tried to purify the substance responsible for the hypoglycemic action in the extract of RehR (Figure 1A). We found that total sugar level in hemolymph of the hyperglycemic silkworms was decreased by injection of the fraction (Figure 1B). We demonstrated that a major component in the active fraction was polygalactose (Figure 1C). Based on these results, we considered that quality check of the RehR is possible to measure the hypoglycemic activity of various batches of RehR using the hyperglycemic silkworms.

4. Establishment of a simple protocol for evaluation of different batches of the RehR using the hyperglycemic silkworms

For the quality check of herbal medicines, the method should be simple so that the results are highly reproducible, and are able to be performed with low costs. We proposed a new method for preparing the active compound in the RehR extracts. In the previous protocol (Figure 1A), five days with five steps were needed (*18*). Since we previously demonstrated that polygalactose was the active compound in RehR, we tried to establish a new protocol for rapid purification of polysaccharides in the extract of the RehR. From a view of chemical nature of polygalactose, the compound is

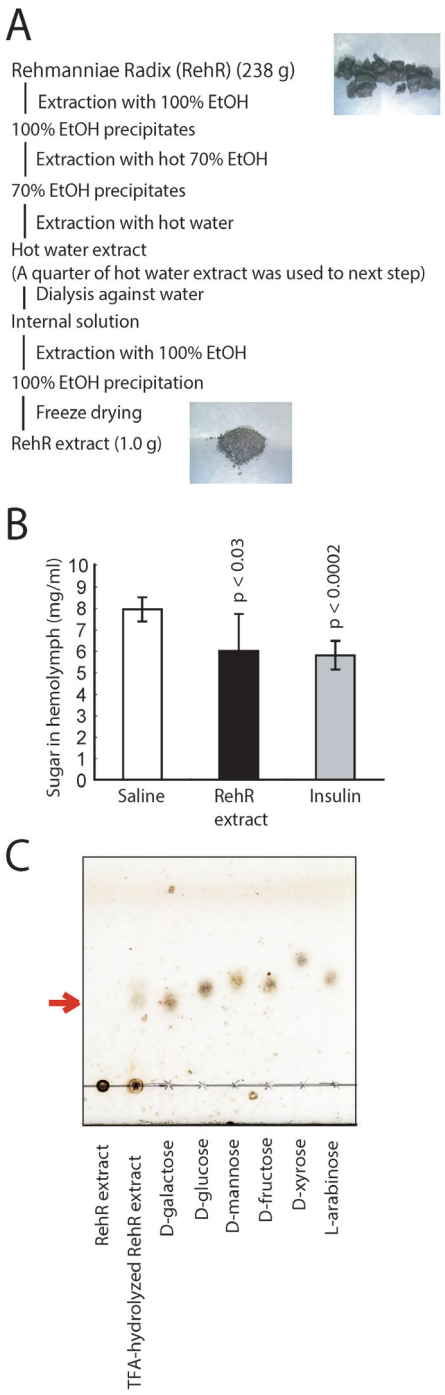

Figure 1. Identification of a hypoglycemic compound in Rehmanniae Radix using hyperglycemic silkworm model. (A) Preparation of a Rehmanniae Radix (RehR) extract. **(B)** Decrease of total sugar level in hemolymph of hyperglycemic silkworms by injection of the RehR extract. **(C)** Analysis of polysaccharides in the RehR extract by thin layer chromatography. Statistical significance between groups was evaluated using Student's *t* test. Figures were taken from Matsumoto *et al.* (*18*) and partly modified.

expected to be separated from monosaccharides such as glucose by ethanol precipitation. Based on this notion, we established a new protocol, which is shown in Figure 2A (*21*). According to the new protocol, we obtained RehR extracts (0.2 g) by ethanol precipitation

from hot-water extracts of 6 different batches of the RehR (5 g) (Figure 2A). A control experiment was done with the same batch (#1) of the RehR extract (extract 1), whose fraction according to the previous protocol (Figure 1A) showed the hypoglycemic activity in the hyperglycemic silkworms (Figure 1B). We prepared the purified fraction by the new protocol (Figure 2A), where the number of steps was reduced from 5 steps to 2 steps. Period needed for preparation was reduced from 5 days to 1 day in the new protocol. The final fraction was dissolved in saline at the concentration of 1 mg/mL and was served to examination of hypoglycemic activity in the hyperglycemic silkworms. The sugar concentration in hemolymph of the hyperglycemic silkworms injected with the purified fraction from batch #1 of the RehR was smaller than that injected with saline, and the difference was statistically significant since the p-value was less than 0.05 by the Student t-test. We next prepared the purified fractions from 5 different batches of the RehR. All of 5 fractions according to the new protocol showed smaller values of the sugar concentration in the hyperglycemic silkworms than the control, however, the differences of extract 4, 5, and 6, were not statistically different since the p-values were larger than 0.05 (Figure 2B). The results suggest that the statistical difference will provide a good evidence

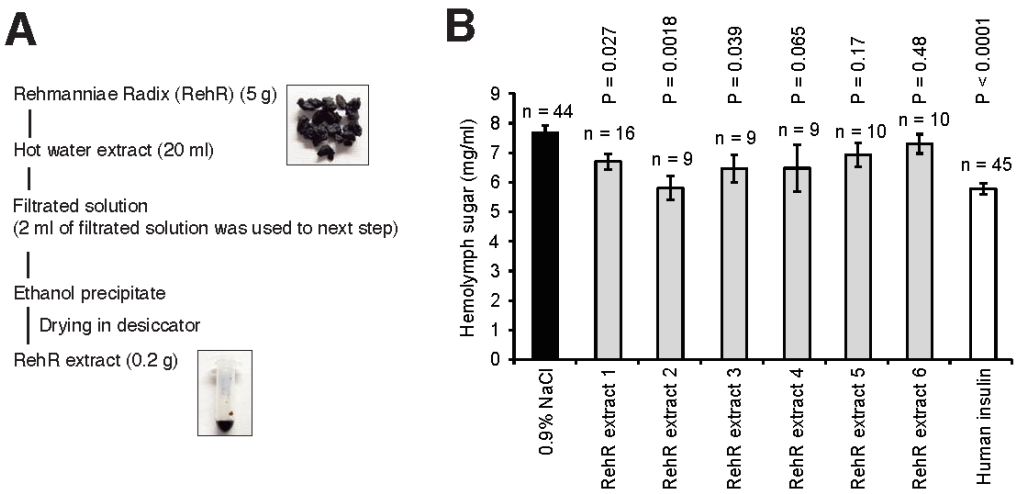

Figure 2. Hypoglycemic activity against hyperglycemic silkworm in different batches of Rehmanniae Radix extracts prepared by a newly established method. (A) Improved method for preparation of Rehmanniae Radix (RehR) extract. **(B)** Decrease of total sugar level in hemolymph of hyperglycemic silkworms by injection of the RehR extracts, which were prepared by the improved method. Statistical significance between groups was evaluated using Student's *t* test. Figures were taken from Matsumoto *et al*. (*21*).

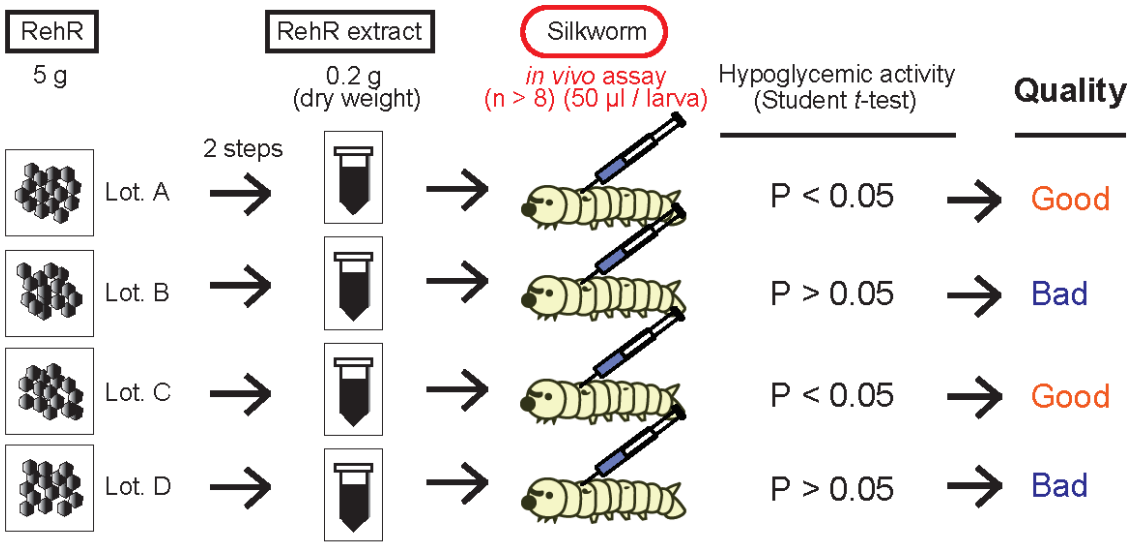

Figure 3. Quality check of Rehmanniae Radix using hyperglycemic silkworms. By comparing total sugar level in hemolymph of hyperglycemic silkworms between sample administration group and saline (control) administration group, $p < 0.05$ in statistically significant difference by Student *t*-test was judged to "good". $p > 0.05$ was judged to "bad".

of RehR for hypoglycemic activity (Figure 3).

5. Versatility of the quality check of herbal medicines using silkworms

For quality check of herbal medicines, mammalian animals have been used for evaluation of efficacies of the herbal medicines. However, mammalian animals such as mice and rats have problems of high breeding costs and ethical issues from the view of animal welfare, it is not suitable for evaluation of large number of the herbal medicines (*22*). Experiments with mammals must be carried out in accordance to 3R that is an international principle, Replacement (developing alternative methods), Reduction (reducing the number of animals) and Refinement (reduce animal suffering) (*23*). Silkworm is expected to be useful as an experimental animal for in vivo evaluation to ensure the quality of herbal medicines. Use of silkworms as an alternative animal are consistent with the idea of Relative Replacement in the 3R. In other words, the evaluation systems using the silkworm are useful alternative experimental systems under restriction of mammalian animal experiments that causes the ethical issues in development and quality check of herbal medicines.

We previously reported that administration of toxic compounds including bacterial toxins caused death of silkworm, and that the LD_{50} values, amounts of components needed for 50% lethality of animals, against silkworms were well consistent to that against mammals (*4,24*). Furthermore, toxicity of compounds at doses smaller than LD_{50} value can be evaluated by monitoring the ALT activity, a tissue damage marker, in the hemolymph of the silkworm (*5*). Therefore, not only the efficacy, but also the presence of toxic substances in the herbal medicines can be tested by *in vivo* evaluation systems using silkworms.

6. Conclusion

In this review, we described that the hypoglycemic activity of RehR, an herbal medicine, can be evaluated by using the hyperglycemic silkworms. By using statistical analysis, we can provide evidence that the extract have the activity. We propose that silkworm is useful for the evaluation of efficacy of herbal medicines by use of lifestyle-related disease models such as hyperglycemia. We also propose type II diabetes model and "humanized silkworm" model such as transgenic silkworm expressing human insulin receptor (*25,26*). We are expecting that the *in vivo* evaluation systems using silkworms will be useful for quality check by monitoring the efficacy of various other herbal medicines than RehR.

Acknowledgements

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Review

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Evaluation of anti-diabetic drugs by using silkworm, *Bombyx mori*

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Summary **Since diabetes patients are increasing in the world, establishment of a novel method for development of anti-diabetic drugs is desired. In this review, we describe recent results of our studies regarding diabetic model using silkworms for evaluation of anti-diabetic drugs for patients of both type I and type II diabetes. The use of the evaluation systems using silkworms is expected to contribute to reduction in cost and in the number of mammals sacrificed for screening of anti-diabetic drugs.**

Keywords: Diabetes, silkworm, *in vivo* evaluation system, anti-diabetic drugs

1. Introduction

Diabetes is a typical life-related disease. Diabetes patients show chronic hyperglycemia, which causes diabetic complications such as retinopathy, nephropathy, and peripheral neuropathy. Diabetes is classified into type I and type II by the difference in mechanism in the onset. Type I diabetes is caused by depletion of insulin, a hormone that regulates glucose level in blood. Type II diabetes is caused by insulin resistance, which occurs either by genetic factors or by life-related diseases such as obesity. In recent years, the number of patients with type II diabetes is increasing in developed countries (*1*). Insulin and drugs improving insulin resistance are clinically used for diabetes patients. Since side effects such as hypoglycemia and obesity by anti-diabetic drugs take place, the development of new therapeutic drugs, which overcome the problem, is desired (*2*).

Several hormones such as insulin strictly regulate blood glucose level in mammalian animals including humans. Those hormones adjust uptake, metabolism, and excretion of sugars in the various tissues in the whole body. Therefore, examination with whole animals is needed to evaluate whether candidates of anti-diabetic drugs have an effect on control of blood sugar level. Since sacrificing many mammals cause problems of high costs and ethical issues in terms of animal welfare, conventional evaluation methods using the mammals such as mice are limited (*3*). To solve these problems, we tried to develop an invertebrate model with silkworms. We previously reported that the silkworm infection models are useful to quantitatively evaluate the therapeutic effects of antibiotics, anti-fungal drugs, and anti-viral drugs (*4-7*). Moreover, we revealed common features between mammals and silkworm in the pharmacokinetics of antibiotics and toxic compounds (*8*). These findings suggested to us that evaluation of therapeutic activities of drugs based on pharmacokinetics using silkworms would be possible. In this review, we introduce our recent findings on the application in the drug discovery by use of hyperglycemic silkworm models.

2. The use of silkworms as model animal for the development of anti-diabetic drugs

Disease models using experimental invertebrates such as *Caenorhabditis elegans* and *Drosophila melanogaster* are developed (*9-14*). Since these organisms are very small, it is not easy to measure the sugar level in their hemolymph and to inject compounds with syringe into the bodies of the animals. In contrast, silkworms are relatively big and move slowly. Therefore, injection experiment into the hemolymph of accurate volume of sample solution using a syringe is easy to be performed (*15*). In addition, silkworm hemolymph in relatively large amounts can be collected. These points are merits of silkworm as compared to *C. elegans* and *D. melanogaster* in the evaluation of anti-diabetic drugs based on the quantitative determination of sugar level

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in the hemolymph. Therefore, we attempted to establish a diabetes model using silkworms and expected that the disease model might contribute to development of anti-diabetic drugs.

3. Evaluation of insulin and AICAR using hyperglycemic silkworms

In a long history of sericulture, mulberry leaves have been used for rearing silkworms. Nutrients contained in the mulberry leaves are absorbed from silkworm intestine to hemolymph and are transferred into the various organs like in mammalian animals (Figure 1A). Silkworms have the organs such as intestine, fat body, and malpighian tubule, which function for exclusion of exogenously administrated chemicals. Moreover, silkworms can maintain glycogen as absorbed carbohydrates in the fat body and the muscle (*16,17*). Therefore, the systems for uptake of sugars and the storage mechanism show common features between silkworms and mammalian animals including humans.

We considered that silkworms could be used for research regarding diabetes by establishing a technique for measuring sugar level in the hemolymph. As the marked difference in the silkworms and humans, a major sugar in hemolymph of the silkworms is trehalose, which is composed with two molecules of glucose (*17*). Silkworms synthesize trehalose by the reaction with trehalose synthase in cells of whole bodies and the resulting trehalose is released into the hemolymph (*18*). Glucose level in the hemolymph of silkworms fed mulberry leaves is very low. Therefore, at the beginning of our study, we wondered whether silkworms become hyperglycemic. In mammals including humans, the blood sugar level is rapidly increased by oral ingestion of sugars such as glucose. We first examined whether glucose level in hemolymph of silkworm would be increased by intake of excess amount of glucose. As a result, we found that total sugar level in hemolymph of silkworms quickly increased within 30 min by intake of an artificial diet containing high amount of glucose (Figure 1B). Moreover, amounts of sugar in fat body, muscle, malpighian tubule, and silk gland in silkworms fed the high glucose diet were much higher than those in silkworm fed a normal diet (Figure 1C) (*19*). Furthermore, we examined conditions whose silkworms become hyperglycemic by monitoring the effect of the feeding time and the amount of glucose in the diet (Figures 1D and 1F). In addition, glucose by itself was detected in hemolymph of the silkworms by intake of the high glucose diet (Figure 1E). These results suggest that a large amount of glucose is directly transferred from intestinal lumen to hemolymph in silkworms. Based on these findings, we expected that we would be able to establish diabetes models with silkworms, where evaluation of anti-diabetes drugs would be possible.

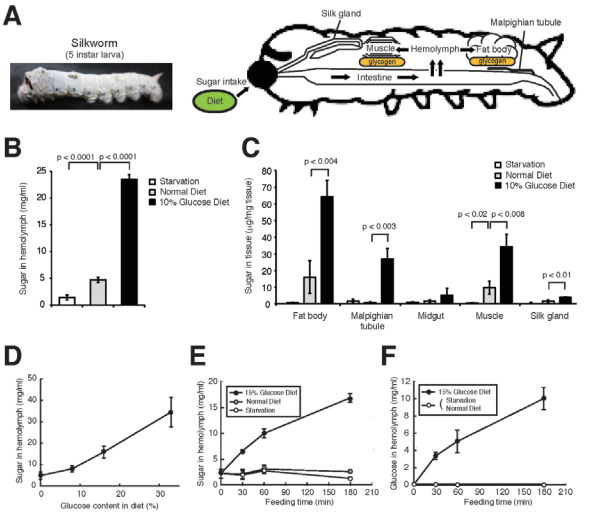

Figure 1. Increase of sugar level in hemolymph and sugar accumulation in various organs of silkworm by intake of high glucose diet. (A) Fifth-instar larvae (left). Transition and distribution in silkworm of dietary sugar (right). **(B)** Increase of total sugar level in hemolymph of silkworm fed a diet containing glucose (10% glucose diet). **(C)** Impact on the accumulation of sugar in various organs (fat body, malpighian tubule, midgut, muscle, and silk gland) of silkworm by intake of 10% glucose diet. **(D)** Effect of glucose content in diet against total sugar level in hemolymph of silkworm. **(E, F)** Immediate increase of total sugar level **(E)** and glucose level **(F)** in hemolymph of silkworm by intake of 15% glucose diet. Statistical significance between groups was evaluated using Student's *t* test. Figures were taken from Matsumoto *et al*. (*19*) and partly modified.

Next we examined whether hypoglycemic activities of anti-diabetic drugs could be observed in the hyperglycemic silkworms. The most typical anti-diabetic substance for treatment of patients with type I diabetes is insulin. In mammals including humans, insulin leads to suppression of gluconeogenesis *via* the phosphorylation of Akt in cells of the liver, adipocytes, and skeletal muscle, resulting in stimulation of glucose uptake followed by decrease in blood glucose level (*20*). Silkworm has bombyxin, a peptide hormone homologous to mammalian insulin. Moreover, the phosphorylation of Akt in the fat body of silkworm was reported to be enhanced by bombyxin (*21*). We hypothesized that silkworm regulates sugar level in the hemolymph *via* activation of insulin-signaling pathway as in the manner in mammals. To test this notion, we asked whether the hypoglycemic activity of human insulin can be observed in the hyperglycemic silkworms. The result demonstrated that the total sugar level in hemolymph of the hyperglycemic silkworms was decreased by injection of human insulin (Figure 2A). We also found that human insulin enhanced the phosphorylation of Akt in cells of fat body of silkworm in an *in vitro* tissue culture system and stimulated glucose uptake into the fat body (Figure 2B) (*19*). These effects of human insulin were suppressed by pre-treatment of wortmannin, which is an inhibitor of the phosphoinositide 3-kinase (PI3K), a key factor of insulin-signaling pathway (Figure 2B). These results suggest that human insulin decreases in sugar level of

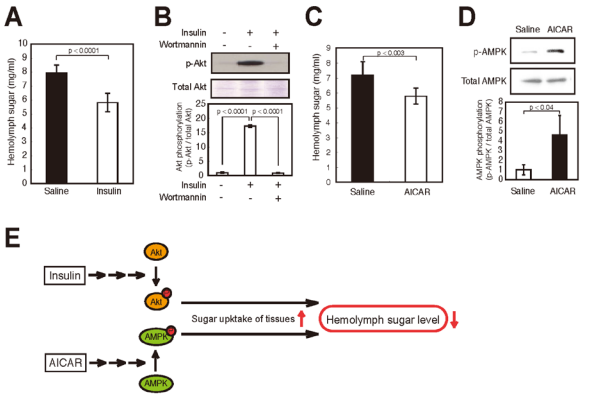

Figure 2. Pharmacological evaluation of human insulin and AICAR by using hyperglycemic silkworm. (A) Decrease of total sugar level in hemolymph of hyperglycemic silkworm by injection of human insulin. **(B)** Increase of Akt phosphorylation in isolated fat body of silkworm by treatment of human insulin in an *in vitro* culture system and inhibition by wortmannin, an inhibitor of phosphoinositide 3-kinase, against the effect of human insulin. **(C)** Decrease of total sugar level in hemolymph of hyperglycemic silkworm by injection of AICAR, an activator of AMP kinase. **(D)** Increase of AMP kinase phosphorylation in isolated fat body of silkworm by treatment of AICAR in an *in vitro* culture system. **(E)** Mechanism of action in silkworm by administration of human insulin or AICAR. Statistical significance between groups was evaluated using Student's *t* test. Figures were taken from Matsumoto *et al*. (*19*) and partly modified.

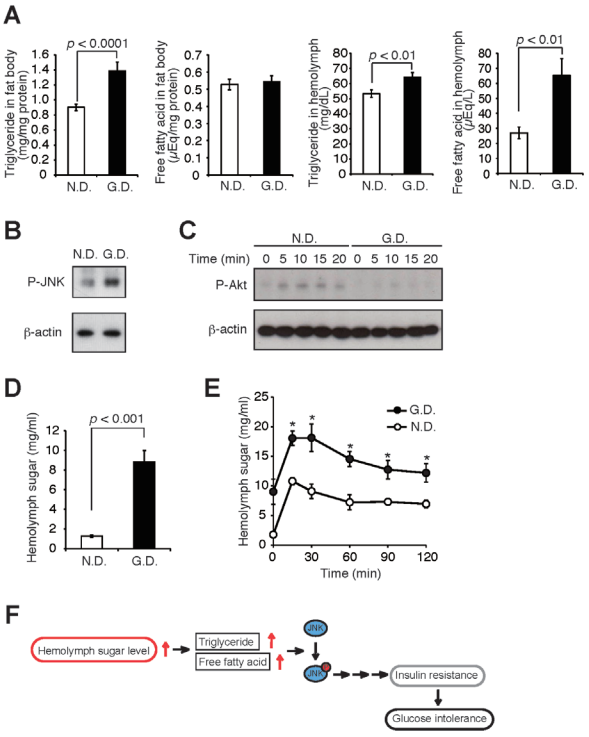

Figure 3. Insulin resistance and glucose intolerance in diabetic silkworm. (A) Accumulation of triglyceride in fat body and increase of triglyceride and free fatty acid in hemolymph of silkworm fed a glucose diet (10% glucose diet) for 18 h. **(B)** Increase of JNK phosphorylation in fat body of silkworm by intake of 10% glucose diet for 18 h. **(C)** Insulin resistance in fat body of silkworm by intake of 10% glucose diet for 18 h. The amount of phosphorylated Akt by insulin was determined by Western blot analysis. **(D)** Increase in fasting sugar level in hemolymph of silkworm by intake of 10% glucose diet for 18 h. **(E)** Glucose intolerance in silkworm by intake of 10% glucose diet for 18 h. **(F)** Mechanism of onset of insulin resistance in the diabetic silkworm. N.D.: normal diet. G.D.: glucose diet. Statistical significance between groups was evaluated using Student's *t* test. $^{*}p < 0.05$. Figures were taken from Matsumoto *et al*. (*23*) and partly modified.

hemolymph in silkworms by enhancing glucose uptake *via* activation of insulin-signaling pathway in cells of tissues including fat body.

In mammals, hypoglycemic effect by activation of AMP kinase (adenosine 5'-monophosphate-activated protein kinase; AMPK) is known as independent system to insulin-signaling pathway. AICAR (5-aminoimidazole-4-carboxamide ribonucleotide) is a compound that activates AMP kinase and promotes to glucose uptake in cells of skeletal muscle in mammalians (*22*). We found that administration of AICAR caused decrease in a sugar level in hemolymph of the hyperglycemic silkworms (Figure 2C). Moreover, the phosphorylation of AMPK in cells of fat body was increased by treatment with AICAR (Figure 2D). These results suggest that AICAR leads to decrease in sugar level in hemolymph of silkworms by activation of AMPK in cells of fat body. Therefore, use of the hyperglycemic silkworms allows us to evaluate anti-diabetic drugs, which activate either insulin-signaling pathway or AMPK pathway (Figure 2E).

4. Evaluation of pioglitazone and metformin using diabetic silkworms

Next, we tested whether hypoglycemic activities of type II diabetes drugs such as pioglitazone and metformin could be evaluated using the hyperglycemic silkworms. The sugar level in hemolymph of the hyperglycemic silkworms, which ate glucose diet for 1 hour, was not decreased by injection of pioglitazone or metformin (*23*). We considered that the hyperglycemic silkworms are not mimicked to type II diabetes model, and attempted to establish a new diabetic silkworm model showing symptoms of type II diabetes. We found that the amounts of triglyceride and free fatty acids in hemolymph of silkworms were increased by intake of high glucose diet for a longer period, 18 hours (Figure 3A) (*23*). Then, we asked whether the hyperlipidemic silkworms under this condition would show symptoms of type II diabetes. Human patients with hyperlipidemia were reported to enhance the phosphorylation of JNK in liver by increase in triglyceride level and free fatty acid level and show insulin resistance (*24*). We demonstrated that the amount of phosphorylation of JNK in the fat body of the hyperlipidemic silkworms was increased (Figure 3B), and an increase in the phosphorylation of Akt in the fat body by human insulin was suppressed (Figure 3C) (*23*). Moreover, the hyperlipidemic silkworms showed higher fasting sugar level in the hemolymph and a decrease in glucose tolerance (Figures 3D and 3F) (*23*). Therefore, we concluded that the

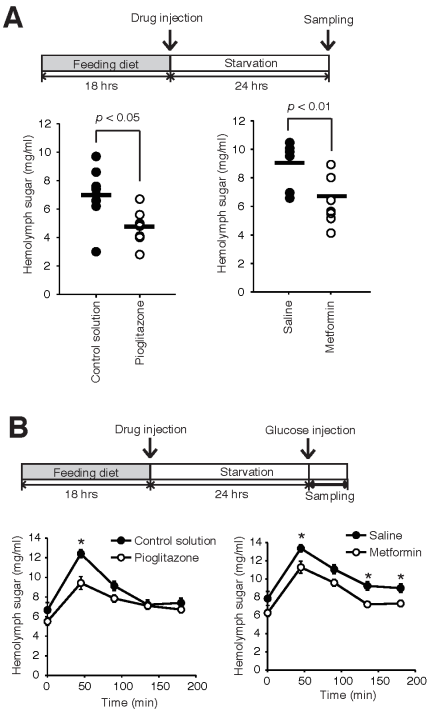

Figure 4. Pharmacological evaluation of pioglitazone and metformin by using diabetic silkworm. (A) Decrease of fasting sugar level in hemolymph of the diabetic silkworm by injection of pioglitazone or metformin. **(B)** Inhibition of decreased glucose tolerance in the diabetic silkworm by injection of pioglitazone or metformin. Statistical significance between groups was evaluated using Student's *t* test. $^{*}p < 0.05$. Figures were taken from Matsumoto *et al*. (*23*).

hyperlipidemic silkworms show the symptoms of type II diabetes.

Next, we tested whether hypoglycemic effects of pioglitazone and metformin can be shown in the diabetic silkworms. As a result, fasting sugar level in hemolymph of the diabetic silkworms was decreased by injection of pioglitazone or metformin, and glucose intolerance was also suppressed (Figure 4) (*23*). These results suggest that the diabetic silkworms are useful for evaluation of hypoglycemic effects of type II diabetes drugs, such as pioglitazone and metformin.

5. Drug discovery of anti-diabetic drugs using silkworm

Our next challenge in future will be how to screen candidates of anti-diabetic drugs using the diabetic silkworms. Since silkworms are suitable for evaluation of therapeutic effects with a large number of individual animals, the evaluation systems by monitoring the therapeutic effects of candidates using silkworms seem to be useful for following subjects: *i*). Identification and purification of active compounds in natural sources such as herbal medicines and foods, *ii*). Screening of effective compounds from chemical libraries, *iii*). Optimization of active compounds by chemical modifications. Some herbal medicines and food are known to have empirically

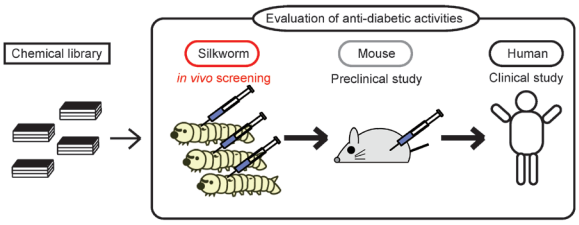

Figure 5. Drug discoveries of anti-diabetic drugs using silkworm.

hypoglycemic effect. However, the active compounds are not yet identified in most cases. Identification by structural determination of the active compounds purified by monitoring the hypoglycemic activities using diabetic silkworms is a conceivable approach. We have reported that polygalactose was identified as an active compound in Rehmannia Radix by monitoring the hypoglycemic activities using the hyperglycemic silkworms (*19*). If large numbers of chemically synthesized compounds are subjected in the evaluation systems using silkworm, it will be possible to select a most active compound having the hypoglycemic activity. The selected compound should be further evaluated in pre-clinical trials using mammalian animal models such as mice, and then would be transferred to clinical trials with human patients (Figure 5). Research using the silkworms is considered to be effective at the first stage before the pre-clinical trials.

6. Conclusion

In this review, we described that the sugar level in hemolymph of the silkworms are increased by intake of high glucose diet. We noted that the silkworm models are useful for evaluation of anti-diabetic drugs for both type I diabetes and type II diabetes. Our study is the first report to propose a screening method of anti-diabetic drugs using invertebrates.

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Review

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Identification of lysocin E using a silkworm model of bacterial infection

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Summary **New antimicrobials with novel mechanisms need to be developed to combat antimicrobial-resistant pathogenic bacteria. The current authors recently reported discovery of a new antibiotic named "Lysocin E". Lysocin E was identified using a silkworm model of bacterial infection. The current review discusses the advantages of using a silkworm model of bacterial infection to identify and develop therapeutically efficacious antimicrobials. This review also discusses the discovery of lysocin E and its novel mechanism of action.**

Keywords: Antimicrobial, lysocin E, silkworm, multidrug-resistant pathogens

1. The antimicrobial crisis caused by the spread of multidrug-resistant pathogens

Due to aging of the population, industrialized nations are now seeing an increase in patients dying due to infectious diseases. Pneumonia is one such an infectious disease, and pneumonia was the 3rd leading cause of death in Japan in 2011. In addition, the spread of multidrug-resistant strains is a serious clinical problem. Recently, the WHO issued a warning that the worldwide spread of multidrug-resistant strains will cause a decrease in clinically useful antibiotics (*1*). New antibiotics with novel mechanisms must be developed to overcome these problems, but far fewer antibacterials have emerged over the past few years than in previous decades. Due to the heightened sense of crisis, governments in the US and Europe have given incentives to pharmaceutical companies to develop new antimicrobials.

2. General problems encountered when developing antimicrobials

One bottleneck in the development of antimicrobials is the decrease in the hit rate of therapeutically efficacious compounds. Thus far, secondary metabolites produced by bacteria in soil have been used to search for drugs, and many antimicrobials have been identified and used clinically in humans. Natural products are still an attractive source in comparison to chemically synthesized compounds because these secondary metabolites are diverse and easier to develop antibiotics from. Indeed, most antimicrobials in clinical use were derived from natural products. If one wishes to develop a new antibiotic, it should at least have a structure unlike that of existing antibiotics since it must be patented to recoup investment. In addition, researchers have been less prone to screen natural products for new compounds because of the vast numbers of attempts to screen those products. Moreover, most compounds that are identified by simply screening for antimicrobial activity usually display poor pharmacokinetics and toxicity to the host. Thus, new antibiotics need to be screened for therapeutic activity in the early stages of drug development. A silkworm model of bacterial infection can indeed facilitate drug development.

3. A silkworm model of bacterial infection for drug development

Kaito *et al*. found that silkworms were killed by injecting hemolymph (the blood of silkworms) with bacteria that are pathogenic to humans such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* (*2*). The current authors used that silkworm model to test the therapeutic efficacy of antibiotics in clinical use. Antibiotics were used to treat silkworms infected with *S. aureus* and their therapeutic efficacy was quantitatively

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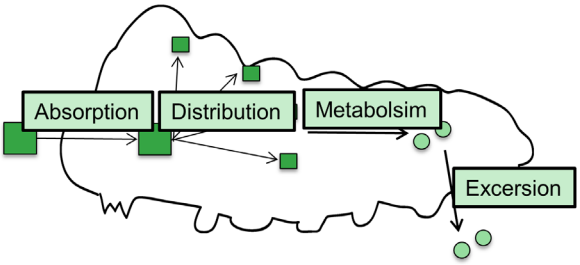

Figure 1. Pharmacokinetics in silkworms. Pharmacokinetics of drug absorption, distribution, metabolism, and excretion in silkworms are similar to pharmacokinetics in mammals.

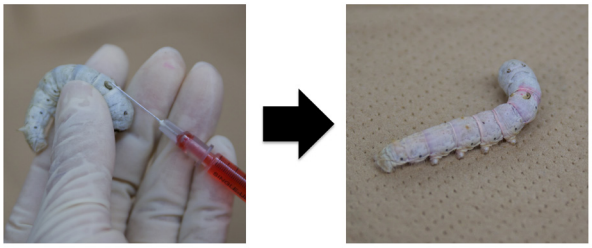

Figure 2. Injection of a sample into silkworm hemolymph. A silkworm is large enough to be handled by hand and the injection volume can be readily controlled because of their slow movement. When red dye was injected into hemolymph (left panel), the dye spread throughout the body (right panel).

Table 1. Therapeutic efficacy of antibiotics in silkworms infected with *S. aureus*

Drugs	ED_{50} (mg/kg • animal)	
	Silkworm	Mouse
Teicoplanin	0.3	0.1
Vancomycin	0.3	1
Minocycline	4	1
Flomoxef	0.2	0.3
Linezolid	9	4
Katanosin B	0.1	0.7

The ED_{50} in the silkworm model and mouse model were similar. This suggests that the therapeutic efficacy of antibiotics can be evaluated using a silkworm model of bacterial infection.

evaluated by calculating the effective dose that produces an effect in half of the animal population taking the substance in question (ED_{50}). The ED_{50} per body weight in the silkworm model was highly consistent with the ED_{50} per body weight in a mammalian model (Table 1) (*3*). Moreover, this correspondence revealed pharmacokinetics in silkworms and mammals were similar. Pharmacokinetics consists of four factors: drug Absorption, Distribution, Metabolism, and Excretion (ADME) (Figure 1). These factors were present in the silkworm model as described below.

Absorption A previous study by the current authors suggested that absorption of small compounds by the mid-gut (the intestine of a silkworm) was affected by the molecular weight and hydrophobicity of compounds consistent to absorption in mammals (*4*). For example, vancomycin cannot be absorbed by the human intestine due to its high molecular weight and low hydrophobicity, and vancomycin similarly displayed no therapeutic efficacy when orally administered to silkworms.

Distribution and Excretion Pharmacokinetic parameters such as distribution and total clearance of several antibiotics in silkworms were similar to the same parameters in mammals (unpublished results).

Metabolism The current authors reported that silkworms have a cytochrome P450 reaction and a conjugation reaction (*5*). When 7-ethoxycoumarin was injected into hemolymph, this compound was metabolized into 7-hydroxycoumarin as a result of the cytochrome P450 reaction and was then transformed into a sugar conjugate form, as well as it is in mammals.

Besides these pharmacokinetic factors, the toxicity of a compound also affects the therapeutic efficacy of antibiotics. In a silkworm model, the lethal doses of cytotoxic compounds were highly correlated with that in rat model, suggesting that silkworms are a suitable model for evaluation of the acute phase toxicity of cytotoxic compounds (*5,6*). These results suggest that therapeutic effect of antibiotics on silkworm infection model is reflected by pharmacokinetics and cytotoxic effect, which mimics those in mammals (*7*). In other words, a silkworm model allows the evaluation of samples that may potentially become drugs.

4. Using a silkworm model of bacterial infection to screen new antibiotics for their therapeutic efficacy

Silkworms are inexpensive and present no ethical issues because they have been used in sericulture for over 4,000 years. In addition, silkworms are large enough to handle by hand and their slow movement allows quantitative injection (Figure 2). These features allow silkworms to be used to screen new antibiotics for their therapeutic efficacy. Choosing what to screen is important when screening for novel compounds. The current authors focused on natural products from bacteria supernatants. In general, re-isolation of same antibiotics from natural products by conventional methods, it tends to have been already saturated, however, we thought it would be possible to obtain novel therapeutically effective antibiotics if we screen by therapeutic effectiveness against silkworm model and used an own isolated library. Thus, a silkworm model of bacterial infection was used to screen the supernatant of bacteria in soil for therapeutic efficacy (Figure 3). In total, 14,651 strains of bacteria were collected from various regions of Japan and 2,794 supernatants displayed antibacterial activity against MRSA *in vitro*. These antibacterial supernatants were tested in a silkworm model of *S. aureus* infection, and ultimately 23 samples were found to display therapeutic efficacy. One of the soil bacteria produced "Lysocin E" (*8*).

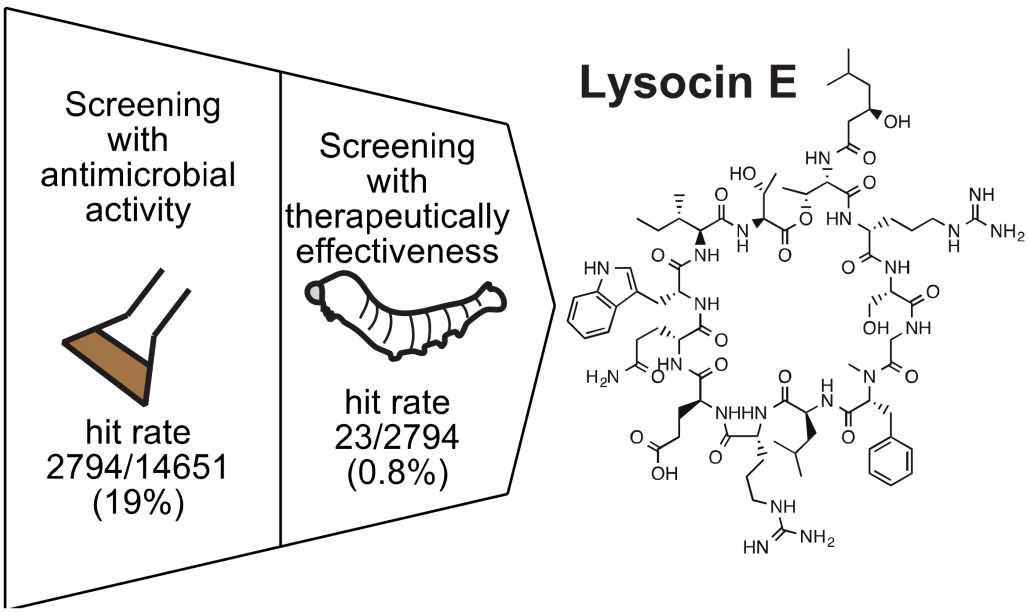

Figure 3. Strategy to screen for new antibiotics. First, samples that displayed antimicrobial activity against MRSA were screened for. Samples that were therapeutically efficacious were then screened again using a silkworm model of bacterial infection. A culture supernatant of bacteria in soil produced a new antibiotic named "Lysocin E".

5. Identification of lysocin E and determination of its structure

A supernatant of a *Lysobacter* sp. collected in Okinawa displayed potent therapeutic efficacy in a silkworm model of *S. aureus* infection despite its weak antimicrobial action. This therapeutically efficacious antibiotic was purified for evaluation in the silkworm model of bacterial infection. During purification, antimicrobial activity increased only 5-fold but therapeutic efficacy increased 300-fold (Table 2). This finding suggests that compounds that displayed only antimicrobial activity were efficiently removed during purification. It is usual case that amount of therapeutically effective compounds in culture supernatants is much smaller than the compounds that only inhibit microbial growth *in vitro*. Previous purification systems only discerned antimicrobial activity, so antimicrobial compounds with no therapeutic efficacy were identified. Now, however, therapeutically efficacious compounds can be efficiently identified using the silkworm model of bacterial infection. Indeed, nosokomycin, a new member of the moenomycin family of antibiotics, was identified using this model (*9*). New therapeutically efficacious compounds can be identified using the silkworm model of bacterial infection.

After purification, the structure of peak E was determined with amino acid analysis and mass spectrometry. The mass spectrum of peak E was distinct from the spectra for other natural products, so the purified compound was further analyzed with NMR and MS/MS. Results indicated that the antibiotic has a novel structure consisting of 12 d- and l-forms of amino acid residues with a short fatty acid chain (Figure 3). This

Table 2. Purification of lysocin E from culture supernatant

Items	ED_{50} (μg/g)	MIC (μg/mL)
Acetone extract	90	25
Butanol extract	4	0.6
Water precipitation	1.8	N.D.
ODS column chromatography	0.5	N.D.
Additional chromatography with an ODS column	0.3	5

During the purification of lysocin E, the reduction in the ED_{50} was much higher than the reduction in the MIC. This suggests that the acetone extract contained antimicrobial compounds that were not therapeutically efficacious in the silkworm model of bacterial infection.

antibiotic was named “Lysocin E” in accordance with the standard system of antibiotic nomenclature. The most difficult aspect of determining the structure of lysocin E was ascertaining its absolute configuration. The chirality of each amino acid was determined with the exceptional 2D-HPLC system developed by Hamase *et al*. (*10,11*). The chirality for glutamine and glutamate could not be determined with heat treatment of lysocin E under acidic conditions because they were hydrolyzed into glutamate by the treatment, but both D and L forms of glutamate were detected. This problem was resolved by Urai *et al*., who developed a new method to distinguish chirality using Hofmann rearrangement. Urai *et al*. are preparing a manuscript that describes how the structure of lysocin E was determined in detail.

6. Mechanistic analysis of lysocin E

Lysocin E displayed antimicrobial activity against

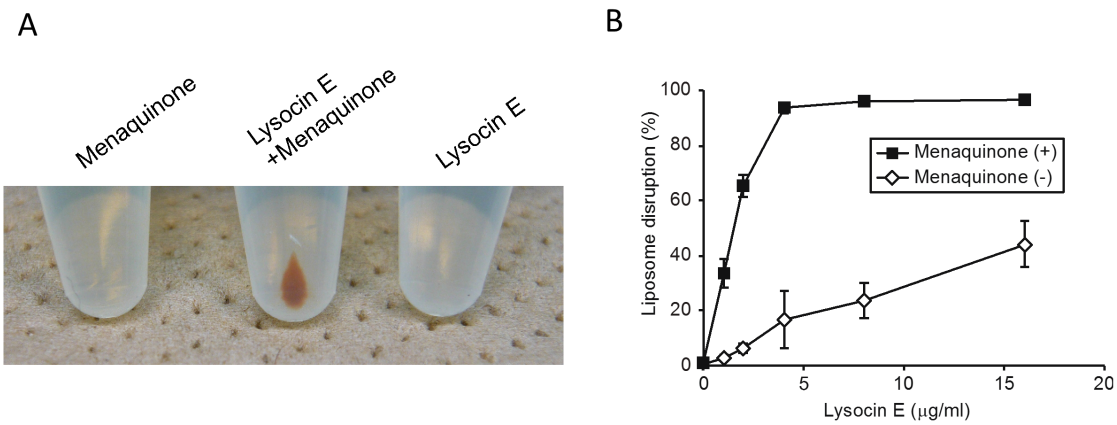

Figure 4. Mechanistic analysis of lysocin E. (A) Mixing lysocin E with menaquinone resulted in a red precipitate. **(B)** Lysocin E specifically disrupted menaquinone containing liposomes.

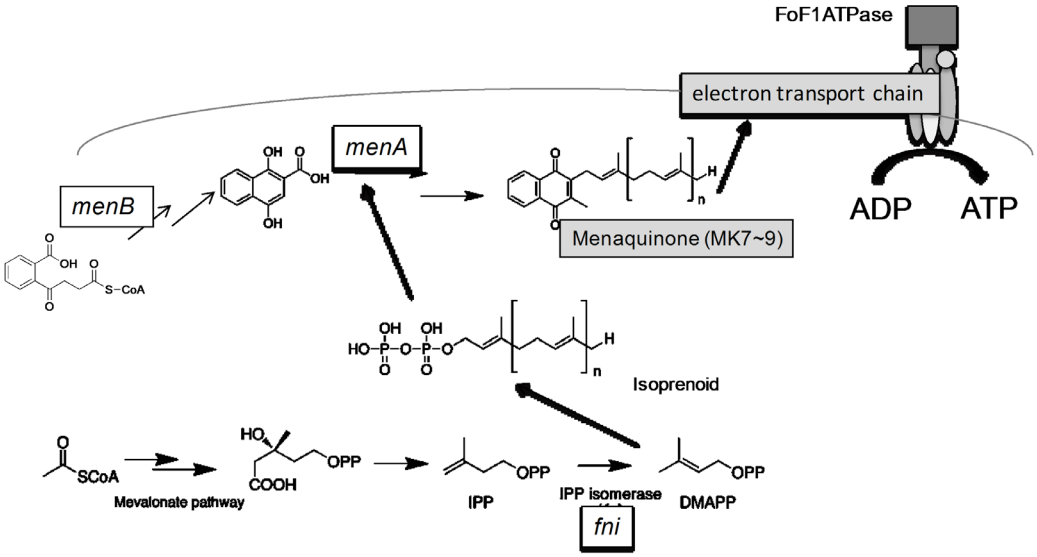

Figure 5. Diagram of the menaquinone synthesis pathway. Menaquinone is a cofactor in the respiratory chain of *S. aureus*, where it is involved in the synthesis of ADP into ATP by F_oF_1 ATPase. Mutants with a knocked out *menA* gene and *menB* gene completely lost the ability to synthesize menaquinone and were less sensitive to lysocin E.

some Gram-positive bacteria, including MRSA, and it demonstrated strong bactericidal activity against *S. aureus* in a brief period of time. This bactericidal activity was associated with membrane damage. In addition, lysocin E has a structure that differs considerably from that of other antibiotics with bactericidal activity. Thus, lysocin E was assumed to have a novel mechanism of action and different targets. Lysocin E-resistant and temperature sensitive-mutants were isolated to reveal the mechanism of action of lysocin E, and gene mutations were found in the menaquinone synthesis pathway. Furthermore, a precipitate was produced by mixing lysocin E with menaquinone (Figure 4A). In addition, the antimicrobial activity of lysocin E was inhibited by addition of menaquinone in an assay. These findings suggest that lysocin E binds to menaquinone directly rather than inhibiting biological processes performed by proteins involved in menaquinone synthesis or the electron transport chain (Figure 5). This hypothesis was confirmed by microcalorimetry, which revealed that lysocin E interacted specifically with menaquinone, a bacterial co-factor in the electron transport chain, and not with ubiquinone, a mammalian cofactor. In addition, lysocin E was found specifically disrupt membranes containing menaquinone (Figure 4B) and to disrupt genes required for menaquinone synthesis. Mutants in which these genes were knocked out were highly resistant to lysocin E. The total synthesis of lysocin E was described by Murai *et al*. (*12*). They synthesized an enantiomer form of lysocin E and it displayed similar antimicrobial activity against *S. aureus*. Menaquinone is an achiral compound, so this phenomenon is theoretically reasonable and it is evidence indicating the substances that lysocin E targets. Therefore, lysocin E was concluded to target menaquinone on the

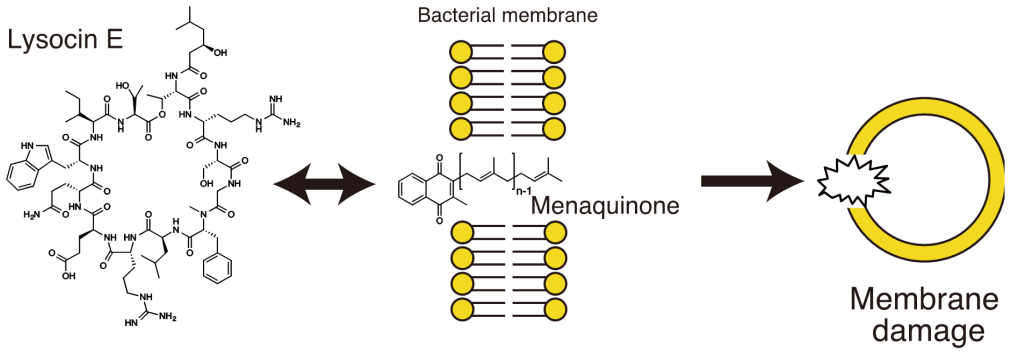

Figure 6. Diagram of the bactericidal action of lysocin E. Lysocin E binds to menaquinone on the surface of *S. aureus* and disrupts its membrane integrity.

bacterial membrane, and interaction between lysocin E and menaquinone would result in the rapid killing of bacteria (Figure 6).

7. Clinical usefulness of lysocin E

Lysocin E was screened for its therapeutic activity in a silkworm model of bacterial infection, so lysocin E would reasonably be expected to display therapeutic efficacy in a mouse model of systemic infection. Lysocin E displayed more potent therapeutic efficacy than vancomycin in a mouse model despite lysocin E having less antimicrobial activity than vancomycin. In addition, injection of lysocin E into the mouse abdomen at more than 500 times the ED_{50} did not kill mice. Furthermore, lysocin E did not cause any organ toxicity after it was repeatedly administered to mice. These features suggested that lysocin E could be useful in clinical treatment of humans, so pre-clinical tests are underway.

Another important factor is the emergence of resistant strains. Lysocin E targets the final product of menaquinone synthesis. Menaquinone is an essential cofactor in the respiratory chain of *S. aureus*. A respiratory chain is required for efficient synthesis of ADP into ATP by F_oF_1 ATPase, so a lack of or a reduction in menaquinone will cause slow growth and decrease the potential pathogenicity of bacteria. Thus, a lysocin E-resistant strain would presumably not be more drug-resistant, but further analyses are required.

Acknowledgments

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Review

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Understanding of bacterial virulence using the silkworm infection model

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Summary **We have used silkworms, larva of *Bombyx mori*, to investigate host-pathogen interactions. Silkworms have several advantages, such as high availability of a large number of animals and ease of injection of quantitative amounts of samples. Human pathogenic bacteria such as *Staphylococcus aureus*, *Streptococcus pyogenes*, or *Pseudomonas aeruginosa* kill silkworms. In this review, I would like to summarize our approach identifying *S. aureus* virulence factors by using the silkworm infection model.**

Keywords: *Bombyx mori*, *Staphylococcus aureus*, virulence factors, infection model

1. Introduction

Staphylococcus aureus is a human pathogenic bacteria causing various diseases. Especially, methicillin-resistant *S. aureus*, MRSA, has afflicted humans from its emergence in the 1960's. In USA, death by MRSA is over 18,000/year, which is more than those by AIDS (*1*).

S. aureus produces various factors in the human body causing diseases, including defensive factors against the host immune system, adhesive factors to host tissues, and toxins that destroy host tissues. Expression of these various virulence factors is regulated by several virulence regulatory factors. *S. aureus* has 16 species of two-component systems that are composed of the sensor protein detecting environmental stimuli and the response regulator acting as a transcription factor (*2*). In these two-component systems, association with *S. aureus* virulence has been reported in the *agr* system (*3*), which acts in quorum-sensing, the *arlRS* (*4*), and *saeRS* (*5*), both of which recognize unidentified signals, and the *graSR* (*6,7*), which is involved in resistance against antimicrobial peptides. In addition, there are many transcription factors including SarA family proteins that regulate the expression of *S. aureus* virulence factors (*8,9*). Identification of novel virulence factors other than these known virulence factors is important for understanding the whole picture of the regulatory network for *S. aureus* virulence factors.

In the past, *S. aureus* virulence factors have been identified by transposon mutagenesis (*10,11*). Most transposons have a tendency to be integrated into some specific DNA sequence, resulting in a biased mutant library (*11*). Using a mutant library constructed with a popular transposon is assumed not to be effective to identify novel virulence factors, since it contains many mutants of known virulence factors. In the recent two decades since the completion of many genome projects, targeting of all genes in monocellular model organisms such as *Escherichia coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, and *Saccharomyces pombe* has been accomplished to reveal the functions of all genes (*12-15*). Such reverse genetic approaches are effective to identify novel gene functions, because there is no opportunity to spend efforts to handle mutants of known genes. We have tried to identify novel virulence factors based on the *S. aureus* genome information (*16*).

2. Establishment of silkworm infection model for human pathogenic bacteria

To identify bacterial virulence factors, it is essential to evaluate the virulence of gene-knockout mutants in animal infection models. Since early times, mammals have mainly been used as infection models for *S. aureus* (*10,17,18*). It is difficult, however, to use large numbers of mammals for infection experiment because of cost and ethical problems. Especially, for the purpose of screening virulence-attenuated mutants from gene-

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knockout mutant libraries, it is needed to use animals other than mammals so that we can use large numbers. We focused our attention on silkworms, moth larva, *Bombyx mori*, a lepidopteran species. Silkworms have been utilized by humans to produce silk for more than 4000 years and thus a great amount of technological information.about the insect has been accumulated. In addition, the silkworm is a solitary insect that has been capitalized on by humans, and has various handy characteristics such as, no biting and no escaping. Before we utilized the silkworm as an infection model for human pathogenic bacteria, *Caenohabditis elegans*, an invertebrate animal, has been used as an infection model for *P. aeruginosa*, a human pathogenic bacteria (*19*). Since the genetic analysis method has been established in *C. elegans*, it is useful to investigate host factors using *C. elegans*. *C. elegans* is, however, a small size animal and is not suitable for injection of quantitative amounts of bacterial solution and for quantitative evaluation of bacterial virulence. In contrast, the 5th-instar silkworm is around 5 cm long, has 700 μL of hemolymph (*20*), and we can easily inject 50 μL solution into the hemolymph by using a tuberculin syringe equipped with a 27 gauge needle (*6,21*). This enabled a quantitative evaluation of bacterial virulence as lethal dose 50% (LD_{50}) (*22*). Furthermore, silkworms are resistant to 37˚C, the human body temperature, and can be used for infection experiments at 37˚C (*23*). Insects, including silkworms, have innate immune systems conserved with mammals. In addition, silkworms have a primed immune system that has several characteristics that resemble acquired immune systems in vertebrates (*24,25*).

Injection of human pathogenic bacteria, such as *S. aureus*, *S. pyogenes*, *P. aeruginosa*, *Vibrio cholerae*, and enteroharnollogic *Escherichia coli*, killed silkworms (*26,27*). *S. aureus* cells injected into the silkworm hemolymph have proliferated in the hemolymph. Injection of antibiotics suppressed silkworm death caused by *S. aureus*, indicating that bacterial proliferation in hemolymph is required for the killing of silkworms. On the other hand, non-pathogenic bacteria against humans such as *Bacillus subtilis* or laboratory strains of *Escherichia coli* did not kill silkworms. These results indicate that virulence of bacteria against humans is reflected in the silkworm infection model.

3. Identification of novel virulence factors of *S. aureus*

S. aureus genome contains 589 gene products that are conserved among bacteria but the functions have not been revealed, which are called "conserved hypothetical proteins" (*16*). We hypothesized that these genes contain novel virulence genes responsible for virulence mechanisms conserved among bacteria. According to the gene-disruption method used in *B. subtilis* by single homologous recombination of a suicide vector into the chromosome (*28*), we tried to construct *S. aureus* knockout strains of these conserved hypothetical proteins (*29*). In *B. subtilis*, it was reported that 200 bp of DNA fragments targeting plasmids homologous to the target DNA sequence in the chromosome causes homologous recombination. In *S. aureus*, we did not obtain gene-disrupted strains by using a 200 bp homologous region in the targeting vector, but obtained gene-disrupted strains by using a 600 bp homologous region. Since the transformation frequency of the *S. aureus* RN4220 strain by plasmids is not much different than *B. subtilis* (*30,31*), there may be a different DNA recombination system between the two bacteria. We constructed gene-disrupted strains of around 100 genes by using a targeting vector harboring around a 600 bp homologous DNA fragment to the target gene. We evaluated the virulence of these gene-disrupted mutants in the silkworm infection model and screened virulence-attenuated mutants. We identified three genes necessary to kill silkworms and named them, *cvfA*, *cvfB*, and *cvfC* (conserved virulence factor) (*32*). These genes also contribute to *S. aureus* virulence in mice and have roles in producing several toxins. To know whether *cvfA* is required for virulence in other bacteria, we examined the *cvfA* function in *S. pyogenes*. We found that *cvfA* is required for *S. pyogenes* virulence in silkworms and mice, and is necessary for *S. pyogenes* production of several toxins including hemolysin (*32*). These results suggest that utilization of the silkworm infection model to evaluate bacterial virulence is a powerful tool to identify novel virulence factors.

4. Functions of novel virulence factors

To reveal the functions of novel virulence factors, we performed biochemical studies based on *in silico* information for protein domains and genetic studies utilizing transcriptome analysis or isolating a genetic suppressor. In the *cvfA*-disrupted mutant, 20% of all gene transcripts were differentially expressed compared with the parent strain (*33*). Downregulated genes in the *cvfA* mutant include *hla* encoding alpha hemolysin, *sarZ* encoding SarA family transcription factor, and *agr* encoding virulence regulator (*34*). Based on the information that CvfA protein has an RNA binding domain and metal-dependent phosphohydrolase domain, we found that CvfA protein has hydrolytic activity against 2',3'-cyclic phosphodiester bond at 3'-terminus of RNA and produces RNA with a 3'-phosphate (*35*). The structural alteration of 3'-terminus of RNA by CvfA conferred resistance against degradation by an exonuclease PNPase (*33*). The phenotype of the *cvfA*-disrupted mutant with decreased hemolysin production was suppressed by disruption of the PNPase gene (*33*). These results suggest that modification of RNA 3'-terminus by CvfA is important for the stability of RNA to regulate the expression of *S. aureus* virulence

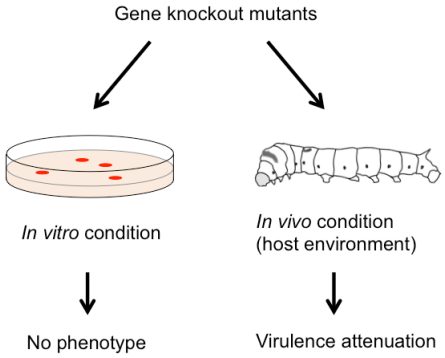

Figure 1. Scheme of phenotypic characterization of gene-knockout mutants. When a gene knockout mutant shows no phenotype under *in vitro* culture condition, more physiological conditions for the bacteria to be exposed should be tested. Silkworm infection model can be used as a host environment to evaluate bacterial virulence properties.

genes. CvfA homolog in *B. subtilis* named RNase Y has an endonuclease activity (*36*). Recently, RNA sequence analysis of the *S. aureus cvfA*-deleted mutant revealed that CvfA acts as an endonuclease against many transcripts (*37*). 2',3'-Cyclic nucleotide at 3'-terminus of RNA is observed at the endonuclease cleavage site of RNA (*38*). The information suggests that CvfA cleaves RNA, produces 3'-phosphorylated RNA, and controls RNA stability.

Biochemical and structural analyses revealed that CvfB protein is an RNA binding protein (*39,40*). The *cvfC* gene regulates expression of a nucleotide synthetase and is required for resistance against detergent (*41*). Recently, we found that ribosomal RNA methyltransferases are required for *S. aureus* virulence *via* conferring resistance against oxidative stress (*42,43*). The novel virulence factors identified from conserved hypothetical genes by using the silkworm-infection model have functions in RNA binding, RNA modification, or nucleotide metabolism, which are different from those of the known virulence regulatory factors such as the two-component systems or transcription factors. Because the gene-disrupted mutants of the novel virulence factors show almost indistinguishable growth from the parent strain, the modification of RNA or alteration of nucleotide metabolism has an important role in the *S. aureus* infectious process in host animals. Molecular mechanisms underlying the requirement of these factors in the host environment should be further clarified in the future.

5. Concluding remarks

We have utilized silkworms as an infection model animal for human pathogenic bacteria and used the model to identify *S. aureus* virulence factors from the conserved hypothetical proteins. Novel virulence factors have been identified and revealed to have functions in RNA modification or nucleotide metabolism. The factors had little report in all organisms, possibly because the phenotype other than virulence is difficult to find. In fact, there are many factors where the gene knockout does not show any phenotypes in model organisms such as *E. coli* (*44-46*). To reveal functions of such factors, it is important to evaluate gene-knockout phenotypes in more physiological conditions than the laboratory culture condition (Figure 1). The method to evaluate bacterial virulence using silkworm infection model is expected to be effective to identify gene functions and contribute to our understanding of bacterial virulence.

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Review

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Recent progress in development of transgenic silkworms overexpressing recombinant human proteins with therapeutic potential in silk glands

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Summary **Since 2000, transgenic silkworms have been developed to produce recombinant proteins with therapeutic potential for future clinical use, including antibody preparations. Lysosomal storage diseases (LSDs) are inherited metabolic disorders caused by mutations of lysosomal enzymes associated with excessive accumulation of natural substrates and neurovisceral symptoms. Over the past few years, enzyme replacement therapy (ERT) with human lysosomal enzymes produced by genetically engineered mammalian cell lines has been used clinically to treat several patients with an LSD involving multi-organ symptoms. ERT is based on the incorporation of recombinant glycoenzymes by their binding to glycan receptors on the surface of target cells and their subsequent delivery to lysosomes. However, ERT has several disadvantages, including difficulty mass producing human enzymes, dangers of pathogen contamination, and high costs. Recently, the current authors have succeeded in producing transgenic silkworms overexpressing human lysosomal enzymes in the silk glands and the authors have purified catalytically active enzymes from the middle silk glands. Silk gland-derived human enzymes carrying high-mannose and pauci-mannose N-glycans were endocytosed by monocytes via the mannose receptor pathway and were then delivered to lysosomes. Conjugates with cell-penetrating peptides were also taken up by cultured fibroblasts derived from patients with enzyme deficiencies to restore intracellular catalytic activity and reduce the excessive accumulation of substrates in patient fibroblasts. Transgenic silkworms overexpressing human lysosomal enzymes in the silk glands could serve as future bioresources that provide safe therapeutic enzymes for the treatment of LSDs. Combining recent developments in transglycosylation technology with microbial endoglycosidases will promote the development of therapeutic glycoproteins as bio-medicines.**

Keywords: Transgenic silkworm, bio-medicine, lysosomal storage diseases, lysosomal enzyme replacement therapy, glycotechnology

1. Introduction

A transgenic (Tg) silkworm *Bombyx mori* was first produced by gene transfer utilizing a *piggyBac* transposon-derived vector in 2000 by Tamura *et al*. (*1*). Since then, Tg silkworms have been developed to produce recombinant human proteins, including procollagen (*2*), cytokines (*3,4*), and monoclonal antibodies (MoAb) (*5-7*), in their cocoons and silk glands. The advantages of Tg silkworms producing recombinant human proteins are: *i*) somewhat larger amounts of recombinant proteins (> 1 mg per larva and/or cocoon) are expressed than those derived from other hosts, *ii*) physical containment of Tg silkworms is easy because the larvae move slowly and seldom

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escape and because the moths cannot fly, *iii*) human pathogens have not been found in *Bombyx mori*, and *iv*) year-round breeding is possible with an artificial diet and expanded facilities, reducing the cost of producing recombinant proteins.

Lysosomal storage diseases (LSDs) are metabolic inborn errors caused by defects in lysosomal enzymes and their related co-factors (*8,9*). LSDs are associated with excessive accumulation of intact substrates in lysosomes due to an enzyme deficiency and exhibit manifestations that are quite heterogeneous. Intravenous (*iv*) enzyme replacement therapy (ERT) has been developed with recombinant enzyme preparations produced by mammalian cell lines, including the Chinese hamster ovary (CHO) and human fibrosarcoma (HT1080) cell lines, that stably overexpress genes that encode human lysosomal enzymes. Since 1991, ERT has been clinically used to treat LSDs, including Gaucher's disease (*10*), Fabry's disease (*11*), Pompe's disease (*12*), mucopolysaccharidoses (MPS) I (*13*), II (*14*), VI (*15*), and Wolman's disease (*16*) involving multi-order disorders such as hepatosplenomegaly, vascular disorders, cardiomegaly, and dysostosis multiplex. *iv* ERT is based on incorporation of a recombinant enzyme *via* cell surface glycan receptors, including the mannose receptor (MR) (*10*) and cation-independent mannose 6-phosphate receptor (CI-M6PR)/insulin-like growth factor type II receptor (*17*), into target organs and their subsequent delivery to intracellular lysosomes to degrade accumulated substrates. However, using *iv* ERT with recombinant human enzyme preparations produced by mammalian cell lines to treat LSDs has several disadvantages, *i.e. i*) large-scale production of recombinant human lysosomal enzymes by mammalian cells is limited and costly in comparison to the large-scale production of therapeutic antibodies (*5-7*) and *ii*) the risk of pathogen infection and the risk of an immune response and adverse effects including production of neutralizing antibodies against enzyme preparations in patients with an LSD and an enzyme deficiency as a result of continuous administration (*18*). Alternative human glycoprotein expression systems need to be developed as more effective, safer, and cheaper lysosomal enzyme sources to overcome these problems. Recently, the current authors succeeded in producing Tg silkworms overexpressing human lysosomal enzymes, including cathepsin A (CTSA) (*19,20*), specifically in the middle silk glands (MSGs) and purifying a catalytically active mature enzyme, allowing elucidation of their crystal structures. The purified enzyme was found to have human-like high-mannose and pauci-mannose N-glycans without insect-specific saccharides. The recombinant human glycoproteins produced by the silk glands of Tg silkworms could be clinically used to treat human diseases as glycobiologics that are slightly antigenic because of their glycan structures.

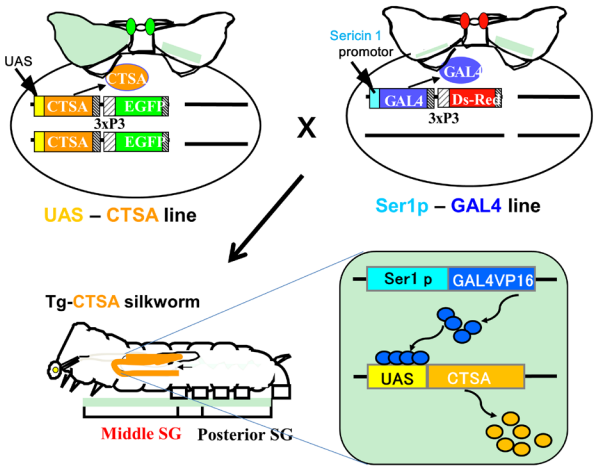

Figure 1. Establishment of transgenic silkworm line overexpressing human lysosomal cathepsin A (CTSA) in the middle silk glands. A Tg silkworm line (Tg-CTSA) overexpressing the human CTSA specifically in the middle silk glands (MSG) was established by crossbreeding between the UAS-CTSA [UAS-CTSA, 3xP3-EGFP] and the Ser1p-GAL4 [Ser1p-GAL4VP16, 3xP3-DsRed2] lines, both are established by utilizing *piggyBac* vectors and fluorescence (due to EGFP or DsRed2) screening of the G1 embryos. In the MSGs of the obtained Tg-CTSA silkworm line harboring both transgenes of the UAS-CTSA and the Ser1p-GAL4VP16, the human CTSA is overproduced under transcriptional control of the MSG-specific sericin 1 gene promotor (Ser1p)–driven GAL4VP16 and the upstream activation sequence recognized by GAL4VP16.

2. Utilizing *piggyBac* vectors to establish transgenic silkworms expressing human lysosomal enzymes

A Tg silkworm line expressing human CTSA in its MSGs (Tg-CTSA) was established as shown in Figure 1. A cloned open reading frame (ORF) fragment of CTSA was inserted downstream of the upstream activation sequence (UAS) of the *piggyBac* vector pBac[UAS-3xP3-EGFP] plasmid (*21*). The MSG-specific sericin I gene promoter (Ser1p) and its 3'-UTR region, including the BlnI site, was amplified from the genomic DNA of *B. mori* with PCR to construct the plasmid pBac[Ser1p-BlnI-UTR, 3xP3-DsRed2] (*22*), and this plasmid was inserted into regions including the *Nhe* I and *Bgl* II sites of pBacMCS (*23,24*). To construct the plasmid pBac[Ser1p-Gal4VP16, 3xP3-DsRed2], a Gal4VP16 *Nhe*I fragment (*22*) was inserted into the *Bln*I site of the plasmid pBac[Ser1-BlnI-UTR, 3xP3-DsRed2]. To generate a transgenic line with UAS-CTSA or Ser1p-Gal4VP16, the *piggyBac* vector pBac[UAS-CTSA, 3xP3-egfp] or pBac[Ser1p-Gal4VP16, 3xP3-DsRed2] was co-injected with the helper plasmid pHA3PIG into pre-blastoderm embryos of the *w-1 pnd* strain (*1*). The hatched larvae were reared on an artificial mulberry diet at 25ºC. G1 embryos were screened under a fluorescence stereomicroscope equipped with an EGFP or DsRed2 filer and a UAS-CTSA or Ser1-Gal4VP16 line was obtained. The UAS-CTSA line was crossed with the Ser1p-Gal4VP16 line, and silkworms (Tg-CTSA) harboring two transgenes, UAS-CTSA and Ser1p-Gal4VP16, were produced.

3. Molecular properties of recombinant human lysosomal enzymes derived from silk glands of the transgenic silkworm

Studies by the current authors have succeeded in functional expression of genes encoding human lysosomal enzymes and posttranslational modification of the MSGs of Tg silkworms. CTSA is a multifunctional lysosomal glycoprotein that exhibits serine carboxypeptidase (cathepsin A) activity as well as protective action to activate lysosomal neuraminidase 1 (NEU1) and stabilize acid β-galactosidase (GLB1) by formation of a multienzyme complex in lysosomes (*19*). In mammalian cell lines, CTSA is biosynthesized as a 54-kDa precursor (catalytically inactive zymogen) and is processed into a catalytically active mature 32/20-kDa form while en route to lysosomes (Figure 2A) (*20*). As shown in Figure 2B, the cathepsin A activity of human mature CTSA at a pH 5.6 towards the artificial substrate Z-L-phenylalanyl-L-leucine was specifically observed in the MSGs but not in the posterior silk glands (PSGs) of the 5th instar larvae of Tg-CTSA harboring two transgenes, UAS-CTSA and Ser1p-Gal4VP16, indicating that expression of CTSA is controlled by the MSG-specific Ser1 promotor. In light of the specific activity (2.7 mmol/h/mg protein) of the human cathepsin A purified from a CHO cell line stably expressing *CSTA* cDNA (*20*), the estimated amount of CTSA expressed in the 5th instar larvae of Tg-CTSA was about 0.1 mg/larva. Approximately one gram of enzyme preparations is reported to be necessary to continuously treat a patient with an LSD per year. An estimated 50,000 larvae are necessary to obtain and purify the same amount of functional human enzymes from the MSGs of Tg-CTSA silkworms.

Immunoblotting with each antibody against the 32-kDa and 20-kDa subunits of mature CTSA (*25*) revealed that three specific immunoreactive bands migrated at 52, 31, and 20 kDa, corresponding to the precursor form and mature subunits, which migrated slightly faster than those (54, 32, and 20 kDa) derived from the CHO cell line stably expressing *CTSA* (20) (Figure 2C). Treatment of the recombinant CTSA proteins with peptide-N-glycosidase F (PNGaseF) revealed the presence of *N*-glycans attached to each precursor and mature subunit, which migrated faster at 50, 30, and 18 kDa, respectively (Figure 2C). These bands were equivalent in size to those of digested DNA from the CHO cell line (Figure 2C). These results indicate that all of the precursor (52-kDa) and mature subunits (31-kDa/20-kDa) expressed in the MSGs carry *N*-glycans.

Recombinant human mature CTSA (cathepsin A) was purified with three-step chromatography utilizing concanavalin A (ConA)-, butyl-, and SP-Sepharose beads. The total cathepsin A activity in the extracts derived from 1,000 larvae was 132 mmol/h. The

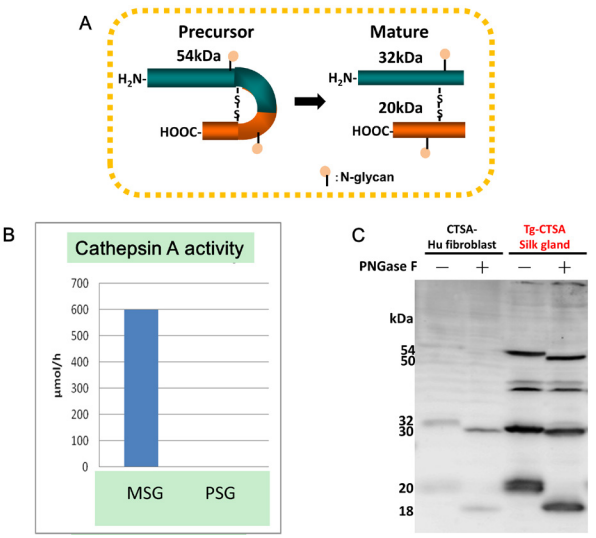

Figure 2. CTSA expression in the middle silk glands of Tg CTSA silkworm. (A) Posttranslational modification of CTSA in mammalian cells. CTSA is biosynthesized in endoplasmic reticulum (ER) as a catalytically inactive 54-kDa precursor (zymogen) and processed to the active 32/20-kDa mature enzyme in the course of transport to lysosomes. **(B)** Cathepsin A activity due to tIne human CTSA was specifically expressed in the middle silk glands (MSGs) but not in the posterior silk glands (PSGs) of Tg-CTSA silkworm under the transcriptional control of the MSG-specific sericin-1 promotor. **(C)** In the MSGs of Tg-CTSA both 52-kDa precursor and 31/20-kDa mature enzyme were expressed as soluble N-glycosylated proteins. Because of their sensitivity to PNGase F. The 52-kDa precursor was considered to be processed to the active 31/20-kDa mature form in the MSGs similarly in mammmalian cells.

total recovery of cathepsin A activity was 18%, and the degree of purification was above 98% (67-fold), according to high-performance liquid chromatography (HPLC)–MS/MS under native conditions.

The N-glycan structures attached to the recombinant mature CTSA purified from the MSGs are summarized in Figure 3 (center). Each mature subunit carries one N-glycan attached to the N-glycan sequon (*19,20*). According to MALDI-TOF-MS, the major *N*-glycan structures were Man3GlcNAc2- (M3b), GlcNAcMan3GlcNAc2- (GNb), Man2Man3GlcNAc2- (M5), GlcNAc2Man3GlcNAc2- (GN2), Man3Man3GlcNAc2- (M6), Man4Man3GlcNAc2- (M7), Man3Man3GlcNAc2- (M8), and Man5Man4GlcNAc2-PA (M9). Most of the major *N*-glycans were high-mannose forms such as M8 and pauci-mannose forms such as M3. The latter are typically found in insects and are also present in mammals as metabolic intermediates. However, fucose residues linked to the core insect-specific α-1,3-linked to GlcNAc residues were seldom detected. The N-glycosylated MoAbs purified from the cocoons of the Tg silkworm have been found to co-express heavy and light chains containing human-like high-mannose N-glycans with no or less insect-specific core-fucose residues (*7*), and these N-glycosylated MoAbs have markedly higher antibody-dependent cellular cytotoxicity as antibody preparations (*5,6*). These

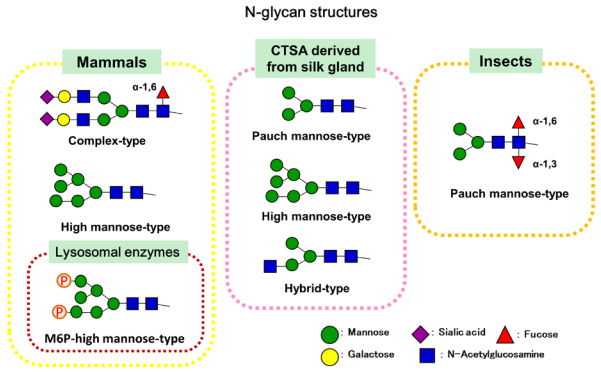

Figure 3. Comparison of N-glycan structures among human, MSG of Tg-CTSA silkworm and insects. In mammals the complex-, hybrid- and high mannose-type N-glycans are present. Lysosomal matrix enzymes have the terminal mannose 6-phosphate (M6P)-carrying high mannose-type (Left bottom). In contrast, insects have pauch mannose-type N-glycans containing insect-specific α-1,3 core fucose as well as α-1,6 fucose residues (Right). The N-glycans attached to the human CTSA in the MSGs of Tg-CTSA silkworms were human-like high mannose- and hybrid as well as pauchi-mannose type (Center), although the terminal M6Ps were not contained in the high mannose-type.

findings suggest the recombinant human glycoproteins produced by the silk glands of Tg silkworms could be clinically used to treat human diseases as glycobiologics that are slightly antigenic because of their glycan structures.

4. Intracellular delivery of mature CTSA purified from silk glands to lysosomes

As described above, mature CTSA derived from the MSGs was found to carry human-like N-glycans with terminal Man and GlcNAc residues. Therefore, purified mature CTSA should be endocytosed by macrophages and monocytes by binding to the cell surface mannose receptor. However, CTSA cannot be readily incorporated *via* CI-M6PR and delivered to lysosomes because CTSA derived from the MSGs does not carry terminal mannose 6-phosphate residues (M6P) in its N-glycans. A study has shown that fluorescein-conjugated mature CTSA at 1 μM can be incorporated into a cultured murine monocyte cell line after incubation at 37ºC for 24 h. Intracellular granular fluorescence due to the fluorescein-CTSA was found to co-localize with that of LysoTracker, which is a late endosome/lysosomal marker (Figure 4). These findings indicate that CTSA can be incorporated *via* the mannose receptor and that the fluorescein-CTSA derived from the MSGs of Tg-CTSA can be delivered to lysosomes.

5. Therapeutic effects of modified CTSA derived from Tg-CTSA on fibroblasts from a patient with galactosialidosis

Over the past few years, cell-penetrating peptides

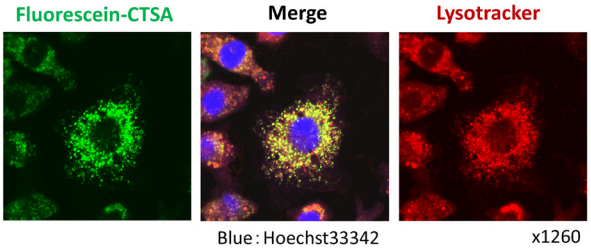

Figure 4. Lysosomal distribution of fluorescein-conjugated CTSA derived from MSGs of Tg-CTSA silkworm in murine monocytic cell line. Fluorescein-conjugated CTSA purified from the MSGs of Tg-CTSA carring terminal mannose-type N-glycans were incorporated into the murine monocytic cell line and transported to lysosomes (Left, green fluorescence), colocalized (Center) with Lysotracker (Right, red fluorescence). Nuclei were stained with Hoechst33342 (Blue). Magnificaion (×1260).

(CPPs) such as oligoarginine vectors have been a powerful tool for intracellular delivery of cargo molecules, including recombinant proteins and nucleic acids (*26,27*). Mature CTSA derived from the MSGs of Tg-CTSA does not carry the terminal M6P residues in its N-glycans, so intracellular uptake of CTSA derived from Tg-CTSA *via* CI-M6PR is not expected. CTSA, NEU1, and GLB activity are deficient in skin fibroblasts derived from a patient with galactosialidosis (GS). Previous studies demonstrated that the recombinant human CTSA produced by CHO cell lines expressing *CSTA* cDNA is taken up *via* cell surface CI-M6PR for delivery and that this recombinant human CTSA partially restores deficient enzyme activity. Oligoarginine (R8)-conjugated CTSA (R8-CTSA) was prepared by mixing BS^3-modified R8 peptides (*26*) with mature CTSA purified from MSGs at 37ºC, and the replacement (therapeutic) effects on a fibroblast cell line from a patient with GS were examined. R8-CTSA but not CTSA itself was found to restore deficient enzyme activity, including deficiencies in CTSA, NEU1, and GLB1, in a dose- and time-dependent manner. Cathepsin A activity recovered to a level equal to that of fibroblasts from a normal subject 24 h after treatment with R8-CTSA (2 μmol/h). The protection of NEU1 and GLB was also found to be partly restored. The current authors previously reported that sialylglycoconjugates, including sialyloligosaccharides, accumulate in fibroblasts from patients with GS (*28*). The addition of R8-CTSA to culture medium caused a marked reduction in the sialylglycoconjugates that accumulated in fibroblasts from a patient with GS according to *Maackia amurensis* (MAM) lectin staining, which recognizes and binds to terminal sialic acid residues of sialylglycoconjugates (*28*). Thus, R8-CTSA can be incorporated into fibroblasts *via* macropinocytosis with an R8 tag as a CPP, it can be delivered to lysosomes, and it can activate NEU1 to degrade accumulated sialyloligosaccharides, suggesting that R8-CTSA would therapeutically benefit patients with GS.

6. Perspectives for the future

Transgenic silkworms producing recombinant human proteins and enzymes in their silk glands are a promising bioresource to provide and promote the development of slightly antigenic therapeutic glycoproteins as safe bio-medicines for the treatment of human diseases, including LSDs. Recently, the current authors established another transgenic silkworm line overexpressing a functional human lysosomal enzyme. That enzyme was produced in the MSGs at an amount of 1 mg of glycoprotein per larva and/or cocoon, but the N-glycosylated enzyme does not carry terminal M6P residues. Genetic engineering will need to add terminal M6P residues to the N-glycans attached to human lysosomal enzymes; glycosyltransferase and/or glycosidase genes could be co-expressed in the MSGs of transgenic silkworm lines. Alternatively, combining recent developments in transglycosylation technology with microbial endoglycosidases is a possibility (*29*). A glycotechnology approach to the chemo-enzymatic synthesis of artificial glycopeptides/glycoproteins (neoglycoproteins) has been developed using N-glycan oxazoline derivatives and the transglycosylation activity of microbial endoglycosidases and their mutants, including endo β-N-acetylglucosaminidases such as Endo-M from *Mucor hiemalis* (*30*), Endo-A from *Arthrobacter protophormiae* (*31*), and Endo-D from *Streptococcus pneumoniae* (*32*). If high-mannose N-glycans carrying terminal M6P residues can be obtained as the N-glycan donor in large quantities, neoglycoenzymes could be produced for ERT to treat patients with an LSD. This could be accomplished through transglycosylation by utilizing the N-glycosylated human lysosomal enzymes derived from the silk glands of transgenic silkworms as acceptors for M6P-carrying N-glycans.

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Mini-Review

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Using silkworms to establish alternative animal models for evaluation of drug-induced tissue injury

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Summary **Evaluation of tissue injury induced by chemicals is crucial to drug development. Mice and rats, which are effectively used to analyze drug-induced tissue injury, present problems in terms of cost and ethical issues. Although alternative methods have been developed using *in vitro* techniques or invertebrates, evaluation of ADME and the size of animals are still issues that need to be addressed. Use of silkworms can resolve these problems. Silkworms have pharmacokinetic characteristics similar to those of mammals. Injection of various hepatotoxic chemicals also leads to elevated alanine aminotransferase (ALT) activity in the hemolymph of silkworms. Furthermore, transparent transgenic silkworms expressing GFP have been produced to facilitate continuous analysis without the need to collect hemolymph. Analyses using this silkworm have indicated that the intensity of GFP fluorescence observed on the body surface of the silkworm decreases in a time- and dose-dependent manner when hepatotoxic chemicals are injected. These results suggest that the silkworms can serve as alternative animal model for evaluation of drug-induced tissue injury.**

Keywords: Drug-induced tissue injury, alternative animal model, silkworm, hepatotoxicity, fluorescence

1. Introduction

Tissue injury induced by chemicals in mammals, including humans, results in the rapid onset of severe dysfunction of the organs involved in detoxification, as exemplified by fulminant hepatic failure (*1*). Therefore, predicting tissue injury, and especially hepatotoxicity, is an important aspect of novel drug discovery.

When novel therapeutic medicines are developed, *in vivo* trials using animal models are essential to predicting toxicity and drug disposition in the human body. Mice and rats are used to evaluate the toxicity of synthesized compounds and natural medicines (*2,3*). However, the use of mammals as experimental models presents a number of problems, such as cost and ethical issues (*4*). *In vitro* assay systems using human hepatocytes have been developed in order to solve these problems (*5,6*). Toxicogenomic systems should be effective at predicting hepatotoxicity based on the expression of various genes that respond hepatotoxicity (*7,8*). However, these *in vitro* assay systems still present problems in terms of the collection of mammalian cells and differences in conditions from those *in vivo*. An alternative animal model is therefore needed to overcome these problems (Table 1).

Although the invertebrates *Drosophila melanogaster* and *Caenorhabditis elegans* are used in experiments in general biology, injection of a precise volume of a sample to determine the LD_{50} in pharmacological analyses is difficult because invertebrates have small bodies (*9,10*). The silkworm has been proposed as an invertebrate model in which to quantitatively administer a drug solution *via* injection with a typical syringe (*11,12*). Another advantage of using silkworms as an animal model in pharmacological research relates to the characteristics of drug metabolism. Silkworms metabolize chemicals *via* processes similar to those found in mammals; in phase I, reactions are catalyzed by cytochrome P450s and metabolites are conjugated in phase II (*13*). The LD_{50} of various cytotoxic compounds in silkworms closely match similar values in mammals

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(*13*). Furthermore, antibiotics have similar therapeutic effects in silkworms and mammals (*11,14,15*). These results indicate that silkworms may be suitable for screening drug candidates that have therapeutic effects in mammals.

There are various methods of evaluating hepatotoxicity induced by drugs (*16,17*). Alanine aminotransferase (ALT) activity in the blood of mammals is frequently measured to evaluate liver function in basic research and routine practice (*18*). The level of ALT

Table 1. Advantages and disadvantages of in vitro and in vivo models for evaluation of drug-induced tissue injury

Model	Advantages/disadvantages	*Ref*
Hepatocytes (*in vitro*)	Advantages: high throughput analysis, cell imaging system, fewer ethical issues. Disadvantages: reproducibility of *in vivo* pharmacokinetics such as ADME, collection of hepatocytes from mammals.	(*5-8*)
Small invertebrates (*in vivo*)	Advantages: high throughput analysis, clarified genetic background, fewer ethical issues. Disadvantages: difficulty of quantitative sample injection.	(*9,10*)
Silkworms (*in vivo*)	Advantages: high throughput analysis, pharmacokinetic characteristics similar to those of mammals, fewer ethical issues. Disadvantages: less knowledge regarding metabolic machinery of extraneous chemicals.	(*20,25*)
Vertebrates (*in vivo*)	Advantages: pharmacokinetic characteristics similar to those of humans. Disadvantages: cost and ethical issues, need for a large amount of samples.	(*4*)

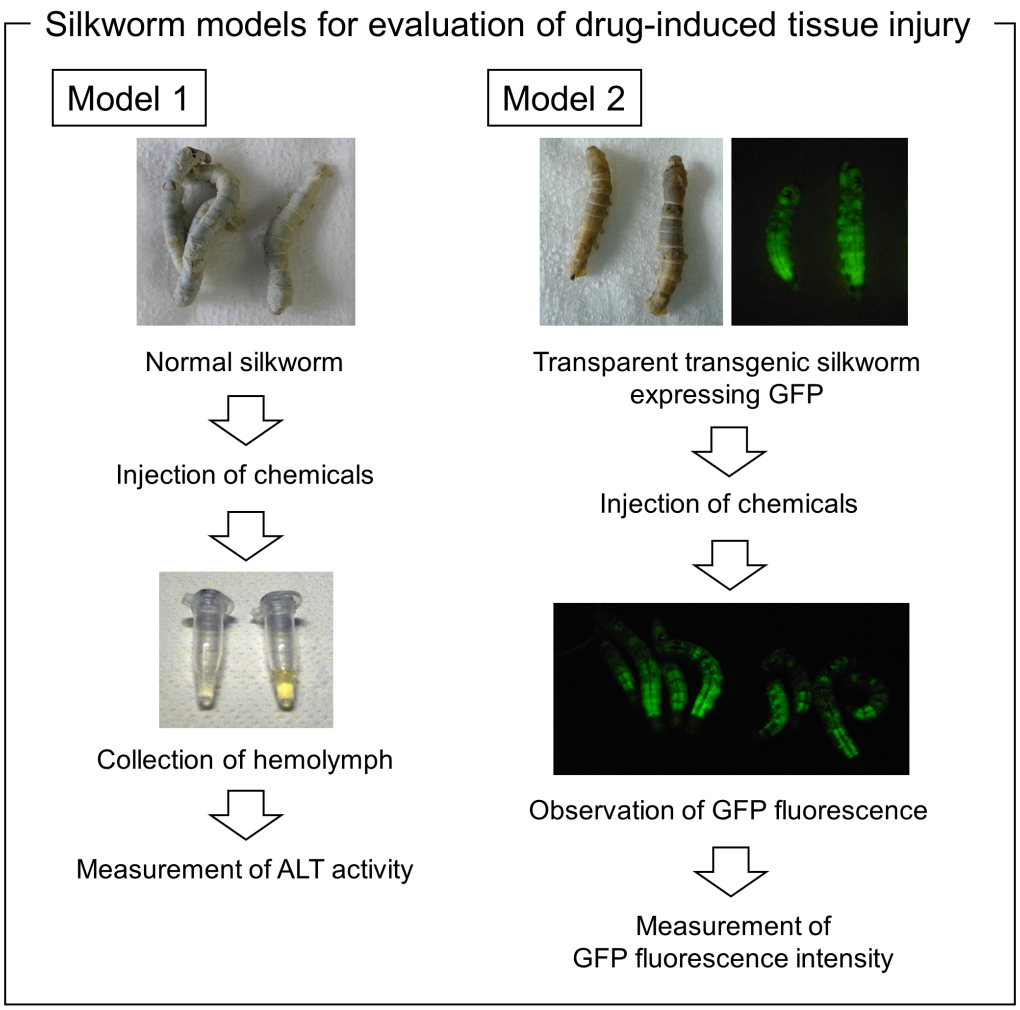

Figure 1. Protocols for silkworm models for evaluation of drug-induced tissue injury. Evaluation of tissue injury in model 1 is based on the level of ALT activity while evaluation in model 2 is based on the intensity of GFP fluorescence.

activity in human blood is considered to be a highly sensitive and fairly specific preclinical and clinical biomarker of cytotoxicity or hepatotoxicity, so many pharmaceutical studies have used the level of ALT in the blood of mammals to evaluate the hepatotoxic effects of natural products or newly synthesized chemicals (*18,19*). Therefore, an alternative animal model that allows evaluation of the level of ALT activity needs to be established. In a previous study by the current authors, ALT activity was measured in the hemolymph of silkworms using usual enzymatic methods (*20*). The ALT level in the hemolymph of silkworm larvae rose with injection of CCl_4, which is generally used as a model compound for examination of hepatotoxic effects in mammals (*20*). Elevated ALT activity in the hemolymph of silkworms has been induced with various hepatotoxic chemicals such as acetaminophen, tetracycline, ketoconazole, and D-galactosamine (*20*). These results suggest that the silkworm can be used to evaluate hepatotoxicity by measuring the level of ALT activity in hemolymph.

As described above, the silkworm model can be used to perform various analyses in pharmacological research, such as evaluations of toxicity and therapeutic action. However, those silkworm models required an invasive approach to obtain hemolymph or to collect tissue. Thus, establishment of a new silkworm model has focused on a model that allows external observation of a silkworm while collecting continuous data from the same animal. Recently, *in vivo* imaging techniques based on fluorescence have progressed markedly and various animal models have been developed (*21-23*). A transgenic silkworm that displays GFP fluorescence has been developed using the GAL4-UAS system (*24*). The fluorescence of the tissue expressing GFP in this transgenic silkworm is consistently visible for a prolonged period as a result of exposure to excitation light. If the tissue expressing GFP is injured by a toxic chemical, the level of fluorescence changes due to the leakage of GFP into the hemolymph. GFP fluorescence has actually been detected in the hemolymph of the transgenic silkworm after the injection of tissue-injuring chemicals such as CCl_4 and salicylic acid (*25*). However, the observation of changes in the intensity of GFP fluorescence from outside the silkworm was hampered by the presence of uric acid. An attempt was made to produce a transparent silkworm to overcome this problem. A previous study by Tamura *et al*. indicated that the body surface of silkworms became translucent upon the intake of allopurinol, an inhibitor of uric acid synthesis, or melamine, an inhibitor of uptake of the uric acid into epithelial cells (*26*). The transgenic silkworm also became transparent when fed a diet containing allopurinol, melamine, and citric acid (*25*). When the transparent transgenic silkworm was exposed to excitation light, GFP fluorescence was clearly observed in the tissue of the fat body (*25*). Furthermore, the intensity of GFP fluorescence in the silkworm decreased with injection of tissue-injuring chemicals (*25*). This decrease occurred in a time- and dose-dependent manner (*25*). These results suggest that the transgenic silkworm expressing GFP can be used to continuously evaluate drug-induced tissue injury.

As described above, the silkworm can be effectively used as an alternative invertebrate model for evaluation of chemical toxicity, infectivity, innate immunity, and virulence (*13,27-29*). The therapeutic effects of antibiotics and anti-hyperglycemic drugs can also be analyzed using silkworm models (*11,30,31*). The present article has described the use of silkworms to evaluate drug-induced tissue injury (Figure 1). The transparent transgenic silkworm expressing GFP may be the first model that allows continuous observation of drug-induced tissue injury *via* a non-invasive means. The use of silkworms will help to reduce the number of mammals sacrificed and streamline drug development.

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Original Article

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Therapeutic effects of three trichothecenes in the silkworm infection assay with *Candida albicans*

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Summary **The silkworm infection assay is a useful method for directly evaluating the *in vivo* therapeutic effects of drug candidates. In the present study, 3 known trichothecenes, trichodermin, epiisororidin E, and verrucarin A, were evaluated as antifungal agents in the silkworm-*Candida albicans* assay. Trichodermin and epiisororidin E yielded effective therapeutic effects, while verrucarin A exhibited no efficacy in this assay system. These results strongly suggest that trichodermin and epiisororidin E are the lead compounds for developing a new antifungal agent.**

Keywords: Antifungal, silkworm infection assay, trichothecenes, *Candida albicans*, cytotoxicity

1. Introduction

Antibiotic candidates against pathogenic microorganisms are generally screened using *in vitro* assay systems such as the paper disk method and microdilution method; however, in most cases, they show no therapeutic effects in *in vivo* assays using mammals because the pharmacokinetics, systemic absorption, and stability of candidates are poor in the mammal body. To overcome these issues, an *in vivo*-mimic infection assay using the silkworm has been introduced to screening at the early stage and is expected to be a relevant strategy for the discovery of therapeutically effective candidates (*1*). In this assay, candidate compounds or test samples are injected into pathogenic microorganism-infected silkworm larvae and the survival rate over a few days is then evaluated. In a silkworm infection model with methicillin-resistant *Staphylococcus aureus* (MRSA), nosokomycins (*2-4*), and lysocins in bacterial culture broths were identified as effective *in vivo* anti-MRSA antibiotics (*1*).

In the course of our screening for antifungal antibiotics using the silkworm infection assay with pathogenic *Candida albicans*, three known trichothecenes, trichodermin (**1**) (*5*), epiisororidin E (**2**) (*6*), and verrucarin A (**3**) (*7*) (Figure 1), were isolated from the culture broths of *Trichoderma* sp. FKI-6009 and *Myrothecium* sp. FKI-5047. Compounds **1** and **2** showed therapeutic effects in *Candida*-infected silkworm, whereas **3** was toxic to the silkworm. In the present study, we described the *in vitro* antifungal activities and cytotoxic and therapeutic activities of **1** to **3** in the silkworm assay. The possibility of trichothecenes as antifungal antibiotics was discussed.

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2. Materials and Methods

2.1. *Materials*

Trichoderma sp. FKI-6009, which was isolated from a soil sample collected in Okinawa main Island, Okinawa, Japan, was used to produce trichodermin (**1**). *Myrothecium* sp. FKI-5047, which was isolated from a soil sample collected in Minato-ku, Tokyo, Japan, was used to produce epiisororidin E (**2**) and verrucarin A (**2**). Miconazol was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2. *Silkworm*

Fertilized silkworm eggs, *Bombyx mori* (Hu•Yo × Tukuba•Ne), were purchased from Ehime Sansyu (Ehime, Japan).

2.3. *Assay to determine in vitro antifungal activity*

The following fungal strains were used in the *in vitro* assay; *Candida albicans* ATCC90029, *C. glabrata* ATCC90030, *C. parapsilosis* ATCC90018,

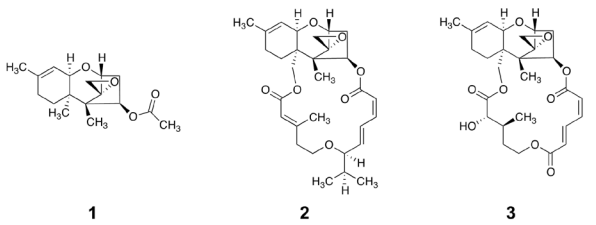

Figure 1. Structures of trichodermin (1), epiisororidin E (2), and verrucarin A (3).

Cryptococcus neoformans ATCC90113, *Aspergillus fumigatus* NBRC33022, *A. flavas* NBRC6343, *A. niger* NBRC105649, *A. terreus* NBRC7078, *Rhizopus oryzae* NBRC4705, *R. microsporus* IFM46417, *R. pusillus* var. *rhizopodiforms* NBRC9744, and *Absidia corymbifera* IFM5335.

Yeast-like fungi including *Candida* spp. and Cryptococcus sp. were subcultured on Potato-dextrose agar (PDA; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) plates at 35°C. Five colonies of ~1 mm in diameter on PDA after a 24-hour incubation at 37°C were suspended in sterile 0.85% saline and the turbidity of the suspension was adjusted at MacFarland 0.5. The suspension was diluted to 1/2,000 in RPMI 1640 medium (Life Technologies, Carlsbad, CA, USA). The minimum inhibitory concentration (MIC) values of **1**, **2**, and **3** against the yeast were determined by the broth dilution assay according to the standard guidelines described in the Clinical and Laboratory Standards Institute (CLSI) documents M27-A3 method (*8*).

Filamentous and Zygomycetous fungi including *Aspergillus* spp., *Rhizopus* spp., and *Absidia* sp. were subcultured on PDA at 35°C until good sporulation was obtained. The sporulating colonies were collected with 1 mL of sterile 0.85% saline, the homogeneous suspension containing spores and conidia was left to stand for 5 min to settle heavy particles, and the upper homogeneous suspension was transferred to a sterile tube. The suspension was adjusted to optical density (OD) at 530 nm, ranging from 0.09 to 0.13 for filamentous fungi and from 0.15 to 0.17 for Zygomycetous fungi. The inocula for the homogeneous suspensions were diluted to 1/50 in RPMI 1640 medium. The MIC values of **1**, **2**, and **3** against filamentous and Zygomycetous fungi were determined by the broth dilution assay according to the standard guidelines described in the CLSI documents M38-A2 method (*9*).

2.4. *Assay for antibacterial activity*

Antibacterial activity against 9 species of bacteria was measured by the paper disk method. Media for microorganisms were as follows: GAM agar (Nissui Seiyaku Co., Tokyo, Japan) for *Bacteroides fragilis*; Waksman agar for *Mycobacterium smegmatis*; Bacto PPLO agar (Becton Dickinson and Company, Franklin Lakes, NJ, USA) supplemented with horse serum 15%, glucose 0.1%, phenol red (5 mg/mL) 0.2%, and agar 1.5% for *Acholeplasma laidlawii*; nutrient agar for *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Xanthomonas oryzae*. A paper disk (i.d. 6 mm, Advantec, Tokyo, Japan) containing 10 µg of a sample was placed on the agar plate. Bacteria, except for *Xanthomonas* oryzae, were incubated at 37°C for 24 hours. *X. oryzae* was incubated at 27°C for 24 hours. Antimicrobial activity was expressed as the diameter (mm) of the inhibitory zone.

2.5. *Silkworm infection assay with C. albicans*

C. albicans TIMM1778 subcultured on a Sabouraud agar plate was inoculated into Sabouraud liquid media and grown for 24 hours at 27°C on a reciprocating shaker at 180 rpm. The culture was diluted in Sabouraud liquid medium at the required concentration (5.0×10^8 cells/mL). The silkworm infection assay with *C. albicans* was carried out by the established method with some modifications (*3*). Hatched silkworm larvae were raised by feeding an artificial diet containing antibiotics (Silk Mate 2S, Nihon Nosan Kogyo, Kanagawa, Japan) in an incubator at 27˚C until the fourth molting stage. On the first day of fifth-instar larvae, silkworms were fed an antibiotic-free artificial diet (Silk Mate, Katakura Industries, Saitama, Japan). On the second day, *C. albicans* (2.5×10^7 cells in 50 µL of Sabouraud liquid medium) was injected into the hemolymph through the dorsal surface of the silkworm using a disposable 1-ml syringe with a 27G needle (TERUMO, Tokyo, Japan). A test compound or sample (in 50 µL of 10% DMSO) was immediately (within one hour of the *C. albicans* injection) injected into the hemolymph, and silkworms were maintained in an incubator at 27˚C. All *C. albicans*-infected silkworm larvae not administered the drug died within 48 hours. When miconazol (50 µg/larva) was injected under these conditions, all silkworms survived and lived after 80 hours.

2.6. *Assay for toxicity to silkworm larva*

A compound (10-100 µg in 50 µL of 10% DMSO) was injected into the hemolymph of silkworm larvae (n = 5), and larvae were maintained in an incubator at 27˚C. The concentration of a compound at which all larvae died within 3 days was defined as a toxic concentration.

2.7. *Assay for cytotoxicity to Chinese Hamster Ovary (CHO)-K1 cells*

Chinese Hamster Ovary (CHO)-K1 cells were cultured in Ham's F-12 (Sigma-Aldrich, St Louis, MO, USA) supplemented with MEM vitamins (Sigma-Aldrich),

geneticin (300 µg/mL, Life Technologies), and 10% heat inactivated FBS (Biowest, Nuaille, France) at 37°C in a humidified atmosphere of 5% CO_2. CHO cells (5.0×10^4 cells/mL) were treated with a compound for 24 to 48 hours. MTT (5.0 µg/mL, Sigma-Aldrich) was added and cells were incubated for approximately 3 hours (*10*). After the cells were lysed with 40% N,N-dimethylformamide, 20% sodium dodecyl sulfate (SDS), 2.0% acetic acid, and 0.030% hydrochloric acid, the absorbance of the solution was read at 550 nm using POWER WAVE X340 (Bio-Tek Instruments, Winooski, VT, USA).

3. Results

3.1. *In vitro antifungal activity.*

The MIC values of **1**, **2**, and **3** against various pathogenic fungi are summarized in Table 1. The MIC value of clinically used miconazol is also shown for comparative purposes. Compounds **1** to **3** exhibited antifungal activities against the yeast-like fungi, and showed moderate activity against some of the filamentous and Zygomycetous fungi. MIC values showed that the antifungal activities of **1** and **3** were similar while **2** exhibited the weakest antifungal activity. However, miconazol exhibited very potent antifungal activity against all the fungi listed in Table 1.

3.2. *Therapeutic effects in the silkworm infection assay with C. albicans*

Compounds **1**, **2**, and **3** were evaluated in the silkworm-*C. albicans* infection assay ($n = 5$). As shown in Figure 2, all infected silkworms not administered a drug (control) died within 48 hours. When **1** and **2** were injected into silkworms under these conditions, they dose-dependently survived. Compound **2** exhibited the most potent efficacy because all silkworms survived 72 hours after the injection at a dose of 50 µg/larva. Compounds **1** and **2** exhibited no toxic effect on silkworm even at 100 µg/larva, whereas **3** was toxic at 100 µg/larva because all silkworms died soon after the injection.

3.3. *Cytotoxicity to CHO-K1 cells*

Cytotoxic effects on the growth of CHO-K1 cells

Table 1. *In vitro* antifungal activity of 1, 2, 3 and miconazol

Items	1	2	3	Miconazol
Yeast-like fungus				
Candida albicans ATCC90029	0.125	1.0	0.125	0.0039
C. glabtata ATCC90030	2.0	16	4.0	0.0078
C. parapsilosis ATCC90018	0.5	4.0	0.125	0.031
Cryptococcus neoformans ATCC90113	2.0	4.0	2.0	0.125
Filamentous fungus				
Aspergillus fumigatas NBRC33022	> 32	> 32	> 32	1.0
A. flavas NBRC6343	> 32	> 32	> 32	2.0
A. niger NBRC105649	4.0	> 32	32	2.0
A. terreus NBRC7078	> 32	> 32	> 32	1.0
Zygomycetous fungus				
Rhizopus oryzae NBRC4705	> 32	> 32	> 32	0.5
R. microsporus IFM46417	> 32	> 32	> 32	2.0
Rhizomucor pusillus NBRC9744	8.0	16	4.0	1.0
Absidia corymbifera IFM5335	8.0	> 32	8.0	4.0

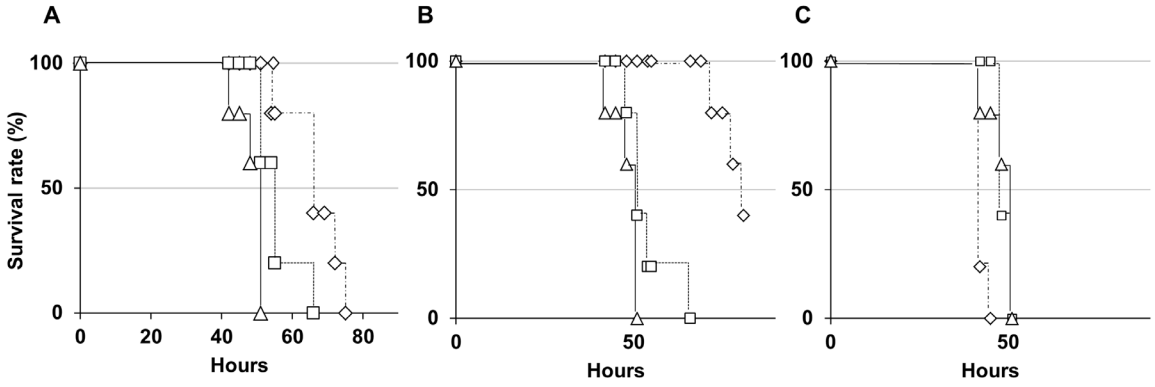

Figure 2. Therapeutic effects of trichodermin (1), epiisororidin E (2), and verrucarin A (3) in the silkworm infection assay with *C. albicans*. (A) 1, (B) 2, (C) 3, ◊: 50, □: 5, Δ: 0 µg/larvae.

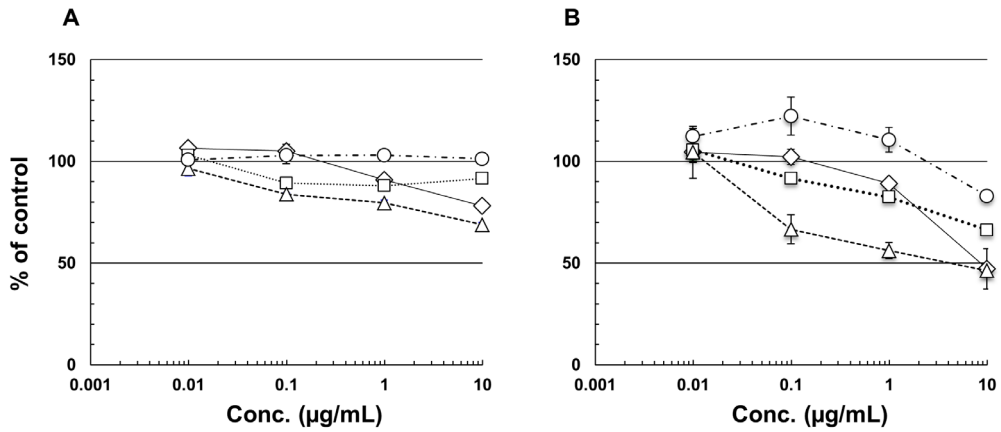

Figure 3. Cytotoxicities of trichodermin (1), epiisororidin E (2), verrucarin A (3), and miconazol (4) in CHO-K1 cells. (A) After a 12-hour incubation. **(B)** After a 24-hour incubation. □◊: **1**, □: **2**, Δ: **3**, ○: **4**.

are shown in Figure 3. After a 12-hour incubation at 10 μg/mL, **1** (22% inhibition), **2** (8%), **3** (31%), and miconazol (0%) exhibited low cytotoxicity. After 24 hours, the cytotoxicities of **1** and **3** were clearly observed with IC_{50} values of 8.8 and 4.2 μg/mL, respectively. However, **2** and miconazol showed low cytotoxicities to CHO-K1 cells (IC_{50}, > 10 μg/mL). These results indicated that **2** showed the lowest cytotoxicity among the three trichothecenes.

3.4. *Antibacterial activity*

The paper disk method revealed that **1** to **3** showed no antibacterial activity at 10 μg/6 mm disk against the 9 bacteria listed in section 2.4.

4. Discussion

In the present study, an *in vivo*-mimic silkworm infection assay with *C. albicans* was performed for the primary screening of antifungal antibiotics, and three trichothecenes, **1** to **3**, were isolated from fungal culture broths. Trichothecenes are generally known as mycotoxins that have toxic effects on eukaryotic cells by inhibiting protein, DNA or RNA synthesis to induce apoptosis (*11,12*). In the present study, the known trichothecenes **1** to **3** exhibited antifungal activities. Although the *in vitro* antifungal activity of **2** was the lowest among the three trichothecenes (Table 1), it exhibited the highest efficacy in the silkworm infection assay with *C. albicans* (Figure 2). These results were similar to those of clinically used fluconazole (FCZ); the *in vitro* antifungal activity of FCZ was previously shown to be less than those of other azoles, but it still exerted beneficial *in vivo* effects because it is metabolically stable and exhibits low plasma protein binding activity (*13*). This may also be the case for the potent therapeutic effects of **2** in silkworm.

The silkworm infection assay may be applied to determining the *in vivo* therapeutic effects and toxicities of candidate compounds. Therefore, trichothecene **2** may be a promising lead for the development of antifungal therapeutics.

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Original Article

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Lactic acid bacteria activating innate immunity improve survival in bacterial infection model of silkworm

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Summary **Lactic acid bacteria (LAB) have been thought to be helpful for human heath in the gut as probiotics. It recently was noted that activity of LAB stimulating immune systems is important. Innate immune systems are conserved in mammals and insects. Silkworm has innate immunity in response to microbes. Microbe-associated molecular pattern (ex. peptidoglycan and β-glucan) induces a muscle contraction of silkworm larva. In this study, we established an efficient method to isolate lactic acid bacteria derived from natural products. We selected a highly active LAB to activate the innate immunity in silkworm by using the silkworm muscle contraction assay, as well. The assay revealed that *Lactococcus lactis* 11/19-B1 was highly active on the stimulation of the innate immunity in silkworm. *L. lactis* 11/19-B1 solely fermented milk with casamino acid and glucose. This strain would be a starter strain to make yogurt. Compared to commercially available yogurt LAB, *L. lactis* 11/19-B1 has higher activity on silkworm contraction. Silkworm normally ingested an artificial diet mixed with *L. lactis* 11/19-B1 or a yogurt fermented with *L. lactis* 11/19-B1. Interestingly, silkworms that ingested the LAB showed tolerance against the pathogenicity of *Pseudomonas aeruginosa*. These data suggest that *Lactococcus lactis* 11/19-B1 would be expected to be useful for making yogurt and probiotics to activate innate immunity.**

Keywords: Lactic acid bacteria, *Lactococcus lactis*, Silkworm, Innate immunity, Infection

1. Introduction

In mammalian innate immunity, dendritic cells and macrophages produce cytokines in response to microbial pathogens (*1*). In insect innate immunity, hemocytes and fat bodies recognize microbial pathogens that induce anti-microbial response (*2*). In silkworms, immune cells produce reactive oxygen species, which activate proteases resulting in cytokine activation. Our group found an active cytokine, paralytic peptide (PP) that induces muscle contraction (*3*). We have screened innate immunity activating substances by using silkworm muscle specimens (*4,5*). This screening method has the following advantages compared to conventional screening using mammalian innate immune cells like macrophages. First, silkworm does not respond to large amounts of lipopolysaccharide (LPS). Second, insect whole body assay reflects ADMET (absorption, distribution, metabolism, excretion, and toxicity). Substances less effective on PK/PD (pharmacokinetics/pharmacodynamics) and/or are toxic on silkworm muscle contraction would be excluded from tests.

Lactic acid bacteria (LAB) have traditionally been used for fermenting dairy foods and probiotics. LAB have the following characteristics, gram-positive, catalase-negative, no spore formation, and are immotile. If an efficient method to select LAB activating innate immunity is established, it would be expected that dairy foods fermented with LAB would be helpful for human health by activating human innate immunity.

In this work, we isolated LAB and evaluated innate-immunity stimulating activity in silkworms. Isolated *L. lactis* 11/19-B1 had high activity in the silkworm contraction assay. We established an efficient method to isolate lactic acid bacteria derived from natural products. We selected a highly active LAB to activate

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innate immunity in silkworm by using the silkworm muscle contraction assay as well. The results revealed that *Lactococcus lactis* 11/19-B1 was highly active on the stimulation of innate immunity in silkworm. Silkworm that normally ingested an artificial diet mixed with *L. lactis* 11/19-B1 or a yogurt fermented with *L. lactis* 11/19-B1 showed tolerance against the pathogenicity of *Pseudomonas aeruginosa*. This LAB would be expected to be probiotics activating innate immunity. These data suggest that *Lactococcus lactis* 11/19-B1 would be expected to be useful for making yogurt and probiotics to activate innate immunity.

2. Materials and Methods

2.1. *Materials*

GAM broth and GAM agar were purchased from Nissui (Tokyo, Japan). MRS broth and MRS agar were purchased from Becton Dickinson (BD, USA). $CaCO_3$-MRS agar was prepared by adding a final 1% $CaCO_3$ concentration (Wako, Osaka, Japan) to MRS agar after autoclaving. ARS (Alizalin red S) milk agar contained 50% (v/v) milk (Meiji, Tokyo, Japan), 1.3% (w/v) agar (Nacalai tesque, Kyoto, Japan), and 0.044% (w/v) Alizalin red S (Wako). Milk was added after autoclaving. AnaeroPak (Mitsubishi gas chemicals, Tokyo, Japan) was used for anaerobic culture on agar plates. Saline was prepared as 0.9% NaCl (Wako). LB medium was prepared with 1% Bacto tryptone (BD), 0.5% Bacto yeast extract (BD), and 1% NaCl (Wako). LB agar plates were prepared as LB medium containing 1.5% (w/v) agar (Nacalai tesque).

2.2. *DNA sequencing*

Fragments containing 16S rDNA was amplified with PCR using KOD FX Neo (Toyobo, Tokyo, Japan), primers 9F and 1541R (*6*), and bacterial colonies. For the amplification of extended 16S rDNA sequence containing a 16S-23S rDNA spacer region, primers 9F (*6*) and 23R (*7*), and genomic DNA were used. Genomic DNA was isolated with a DNeasy Blood & Tissue Kit (QIAGEN, Germany). DNA sequence was determined with direct sequencing, BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA). Sequences were analyzed with NCBI BLASTN 2.2.27+ (*8*), 16S ribosomal RNA sequences database (Bacteria and Archaea, 7,545 sequences). DNA sequences are in preparation for submission to GenBank.

2.3. *Characterization of LAB*

Isolated bacteria were Gram-stained with Gram color (Merck, USA). Bacterial colonies were suspended in 3% H_2O_2 for catalase tests. *S. aureus* RN4220 and *E. coli* JM109 were used as controls. Other identification kits, Api Zym and Api 50 CHL were purchased from Sysmex BioMerieux (Tokyo, Japan), and the data was analyzed with Api web v5.1 database (Sysmex BioMerieux, France).

2.4. *Silkworm muscle contraction assay*

Silkworm muscle contraction was measured as in a previous report (*3*). Briefly, autoclaved bacterial suspension (50 μL) was injected into silkworm muscle specimens. Contraction value was determined as (prelength-postlength)/prelength. Sample amount (mg) giving a 0.15 contraction value is determined as 1 unit.

2.5. *Silkworm infection model*

Silkworm infection model was performed as in a previous report (*9*). Silkworms (*Bombyx mori* Hu•Yo × Tukuba•Ne) were reared at 27°C. Pathogens used in infection model were *P. aeruginosa* PAO1 (*10*), *S. aureus* MSSA1 (*11*), *S. aureus* MRSA4 (*12*), and *E. mundtii* 12/5-1 (*13*) from our laboratory stock. Pathogenic bacteria grown in LB medium overnight were diluted with saline (0.9% NaCl) and injected into fifth instar larva (n = 7) after feeding with Silkmate (Sysmex, Kobe, Japan) overnight. Survival of silkworm larva was counted for five days.

3. Results

3.1. *Isolation and characterization of LAB*

We developed a new and efficient selection medium for milk-fermenting LAB. ARS medium is an agar plate containing milk and Alizalin red S, which is a pH indicator developing a yellow to white color under acidic conditions after milk fermentation.

Samples from natural products containing plants, soil, and digesta of invertebrates such as slug and earthworm were screened to isolate milk-fermenting LAB (Table 1). Each sample of total 697 samples with saline was ground

Table 1. Summary of samples used in this study

Samples	*n*
Earthworm	132
Slug	13
Leach	2
Fruits	168
Vegetable	34
Wild plants	
Fruits	167
Leaf	54
Root	9
Soil	74
Small animals	44
Total	697

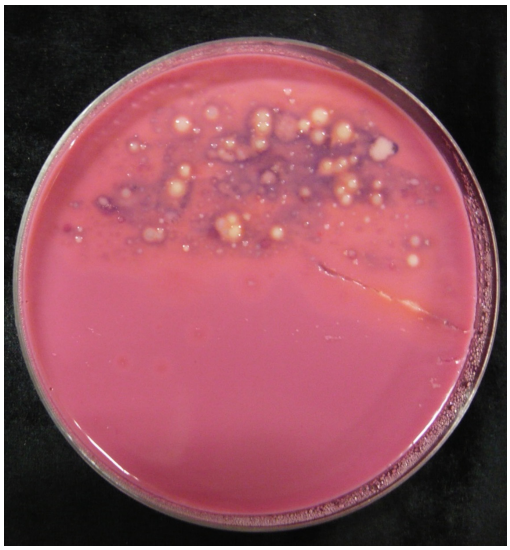

Figure 1. Isolation of LAB on ARS milk agar medium. An example of LAB isolation from soil is shown. Alizaline red S color was changed to yellow to purple by colony formation, which ferment solo nutrient milk.

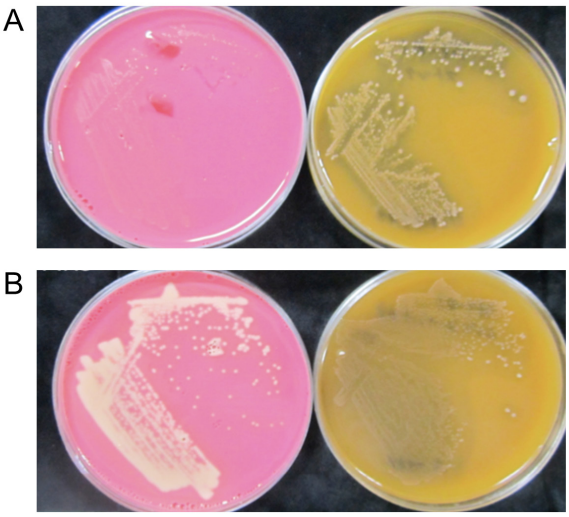

Figure 2. Growth characteristics of LAB. (A) *L. lactis* 11/19-B1 and **(B)** *S. thermopilus* A were streaked on ARS milk medium (left) and $CaCO_3$-MRS agar plate (right).

Table 2. Identification of bacteria and its activity of silkworm muscle contraction assay

Samples	Origin	Identity	ID, %	GenBank accession	Activity (U/mg)
11/19-B1	Kiwifruit	*Lactococcus lactis* IL1403	98	NR_103918.1	105 ± 5[a]
11/28-C3	Kiwifruit	*Streptococcus thermopilus* ATCC 19258	98	NR_042778.1	77
10/30-2	Earthworm	*Lactococcus lactis* IL1403	98	NR_103918.1	45 ± 32[b]
9/10#5	Slug	*Lactococcus lactis* IL1403	98	NR_103918.1	33
12/4-A12	Wild leaf	*Streptococcus salivarius* ATCC 7073	98	NR_042776.1	28
11/21-F1	Wild fruit	*Lactococcus lactis* IL1403	98	NR_103918.1	23
12/3-C11	Wild leaf	*Enterococcus gallinarum* LMG 13129	98	NR_104559.1	7.1
11/28-C8	Apple	*Streptococcus salivarius* CCHSS3	96	NR_102816.1	2.2
11/22-B7	Wild fruit	*Lactococcus lactis* IL1403	99	NR_103918.1	2.0
10/29-10	Earthworm	*Enterococcus casseliflavus*	99	NR_041704.1	0.83
11/27-D5	Soil	*Enterococcus faecium* Aus0004	94	NR_102790.1	0.45
12/3-B11	Pineapple	*Streptococcus salivarius* ATCC 7073	98	NR_042776.1	< 0.3
A	Yogurt	*Streptococcus thermopilus*			43 ± 20[b]
B	Yogurt	*Lactobacillus delbrueckii ssp. bulgalicus* OLL1073R-1			29
C	Yogurt drink	*Lactobacillus delbrueckii ssp. bulgalicus* OLL1073R-1			24
D	Yogurt	*Lactobacillus casei* YIT9029			18 ± 2[b]
E	Yogurt drink	*Lactococcus lactis* JCM5805			17
F	Yogurt drink	*Lactobacillus casei* YIT9029			5.6

[a] mean ± SE (n = 7), [b] mean ± SE (n = 2).

in a mortar and suspended in sterile saline. Supernatant fluids of the suspensions were spread on ARS milk agar. After incubation at 30ºC for two days, more than ten yellow to white colonies were isolated on each plate (Figure 1). We selected Gram-positive colonies from Gram staining colonies on ARS milk agar, since LAB generally recognized as safe (GRAS) are Gram-positive bacteria. Colonies were re-streaked on $CaCO_3$-MRS medium to confirm lactic-acid fermentation (Figure 2). There were 57 samples of lactate-fermenting and Gram-positive bacteria (Table 1). Bacteria subjected to silkworm contraction assays were 23 of the 57 samples (Table 1). LAB was also isolated from commercially available yogurt and silkworm contraction activity was tested for comparison. LAB tested for contraction assays was sequenced for 16S rDNA.

Silkworm contraction activity of LAB is shown in Table 2. LAB isolated from natural samples showed diverse activity from 0.3 to 105 U/mg. *L. lactis* 11/19-B1 showed the highest activity, 105 U/mL, representing higher activity than that of LAB in commercial yogurt tested in Table 2. Other *L. lactis* strains, 9/10#5, 10/30-2, and 11/21-F1 showed relatively high silkworm contraction activity, that is, 33, 45, and 23 U/mg respectively. *Streptococcus strains*, *S. thermopilus* 11/18-C3 and *S. salivarius* 12/4-A12 represented relatively higher activity, 77 and 28 U/mg respectively. LAB already reported to be activating mammal immunity had slightly high activity of silkworm contraction. Commercial yogurt strains, *Lactobacillus delbrueckii* ssp. *bulgalicus* OLL1073R-1 (*14*) and *L. lactis* JCM5805 (*15*), which activate

Table 3. Growth characteristics of *Lactococcus lactis*

Carbohydrate	11/19-B1 (kiwifruit, This study)	KF282 (mustard/cress)[a]	IL1403 (diry)[a]
Glycerol	−	−	−
Erythritol	−	−	−
D-Arabinose	−	−	−
L-Arabinose	+	+	−
Ribose	+	+	+
D-Xylose	+	+	−
L-Xylose	−	−	−
Adonitol	−	−	−
β-Methyl-xyloside	−	−	−
Galactose	±	+	+
D-Glucose	+	+	+
D-Fructose	+	+	+
D-Mannose	+	+	+
L-Sorbose	−	−	−
Rhamnose	−	−	−
Dulcitol	−	−	−
Inositol	−	−	−
Mannitol	+	−	−
Sorbitol	−	−	−
α-Methyl-D-mannoside	−	−	−
α-Methyl-D-glucoside	−	−	−
N-Acetyl glucosamine	+	+	+
Amygdalin	±	+	±
Arbutin	+	+	+
Esculin	+	−	−
Salicin	+	+	+
Cellobiose	+	+	+
Maltose	+	+	+
Lactose	+	+	±
Melibiose	−	−	−
Saccharose	+	+	−
Trehalose	+	+	+
Inulin	−	−	−
Melezitose	−	−	−
D-Raffinose	−	−	−
Starch	−	+	+
Glycogen	−	−	−
Xylitol	−	−	−
β-Gentiobiose	+	+	+
D-Turanose	−	−	−
D-Lyxose	−	−	−
D-Tagatose	−	−	−
D-Fucose	−	−	−
L-Fucose	−	−	−
D-Arabitol	−	−	−
L-Arabitol	−	−	−
Gluconate	±	+	−
2-Keto-gluconate	−	−	−
5-Keto-gluconate	−	−	−

[a] Data from reference 17.

murine macrophages and plasmacytoid dendritic cells respectively showed 24 and 17 U/mg silkworm contraction activity respectively.

L. lactis 11/19-B1 was further characterized for bacterial growth and identification. Growth of *L. lactis* 11/19-B1 was sensitive to temperatures higher than 42ºC. Growth of *L. lactis* 11/19-B1 in MRS medium were stimulated by addition of 0.3% glucose or 0.03% casamino acids. BLAST analysis of 16S rDNA sequence of the 11/19-B1 strain revealed that the 11/19-B1 strain had 98% similarity to *L. lactis* subsp. *lactis* IL1403

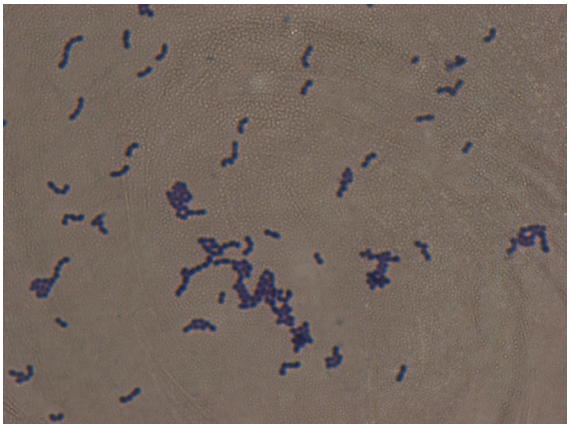

Figure 3. Gram staining of L. lactis 11/19-B1. *L. lactis* 11/19-B1 colony on $CaCO_3$-MRS agar was stained with Gram color (Merck). Image was captured with a CCD camera (Hamamatsu photonics) using an Olympus phase-contrast microscope at ×1,000 magnification.

(NR_103918), *L. lactis* subsp. *hordniae* NCDO2181 (NR_040956) and *L. lactis* subsp. *lactis* NCDO604 (NR_040955). Finally, detailed analysis of extended 16S rDNA sequence (1781 bp) of the 11/19-B1 strain showed 99% similarity to *Lactococcus lactis* subsp. *lactis* IL1403 genome sequence (16, AE005176.1).

Sugar utilization was tested using Api 50 CHL (Table 3). *L. lactis* 11/19-B1 represented a difference compared to *L. lactis* IL1403 in utilization of 6 of 49 sugars and sugar derivatives. L-arabinose, D-xylose, mannitol, esculin and sucrose were positive and starch was negative in *L. lactis* 11/19-B1 as compared to dairy strain *L. lactis* IL1403. On the other hand, *L. lactis* 11/19-B1 represented three-different sugar utilizations with *L. lactis* KF282 derived from plants (*17*). Sugar utilization of *L. lactis* 11/19-B1 matched 77.2% of *Lactococcus lactis* ssp *lactis* 1, and 21.9% of *Lactobacillus brevis* 1 in Api web v5.1 database. A possibility of *L. brevis* being identitical to 11/19-B1 was excluded because of gram-positive bacilli of *L. brevis*, in contrast to gram-positive cocci of 11/19-B1 (Figure 3). Api Zym test was also used for the identification of the strain 11/19-B1 and compared to previous data (*18*) in Table 4. *L. lactis* 11/19-B1 represented 6 and 4 different activities of 19 enzymes as compared to that of *L. lactis* CECT185^T and CECT967^T respectively.

3.2. *Probiotic effect of L. actis 11/19-B1 and its yogurt*

Next, we confirmed milk fermentation with *L. lactis* 11/19-B1 as a starter strain to form yogurt. Commercially available milk was added with 0.03% casamino acids, 0.3% glucose and *L. lactis* 11/19-B1 under sterile conditions, and incubated at 37ºC for 3 days, resulting in yogurt with a clean acid flavor. Control milk with casamino acids and glucose without *L. lactis* 11/19-B1 was not fermented after 3 days. To confirm milk fermentation with *L. lactis* 11/19-B1, colony isolation

Table 4. Enzymatic characteristics of *Lactococcus lactis*

Enzyme	11/19-B1 (This study)	CECT185^T (*lactis* genotype)[a]	CECT967^T (*cremoris* genotype)[a]
Phosphatase alkaline	−	±	−
Esterase (C4)	−	−	±
Esterase lipase (C8)	−	±	±
Lipase (C14)	−	−	−
Leucine aminopeptidase	−	+	±
Valine aminopeptidase	−	−	−
Cystine aminopeptidase	−	−	−
Trypsin	−	−	−
Chymotrypsin	−	−	−
Phosphatase acid	+	+	+
Phosphoamidase	+	+	+
α-Galactosidase	−	−	−
β-Galactosidase	−	±	−
β-Glucuronidase	−	−	−
α-Glucosidase	−	+	−
β-Glucosidase	−	+	+
β-Glucosaminidase	−	−	−
α-Mannosidase	−	−	−
α-Fucosidase	−	−	−

[a] Data from reference 18.

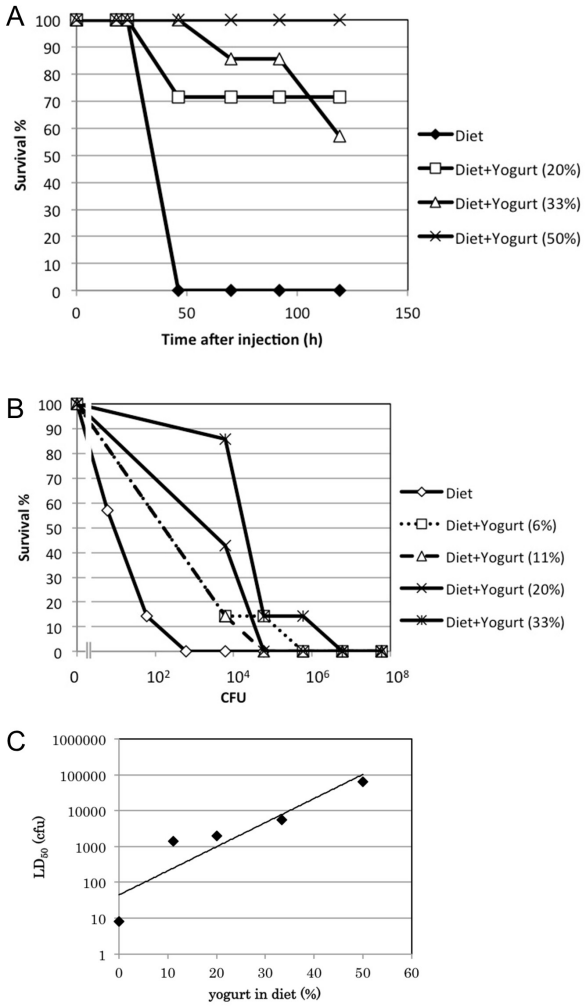

Figure 4. Probiotic effect of *L. lactis* 11/19-B1 yogurt on P. *aeruginosa* infection. (A) Time course of survival of silkworm fed a diet with or without *L. lactis* 11/19-B1 yogurt after *P. aeruginosa* PAO1 infection. **(B)** Dose response of *P. aeruginosa* PAO1 on silkworm survival after 2 days. *P. aeruginosa* PAO1 was injected into 5th instar larva fed a diet with or without *L. lactis* 11/19-B1 yogurt. **(C)** Dose response of *L. lactis* 11/19-B1 yogurt on probiotic effect in *P. aeruginosa*-infected silkworm. Data represented typical one of three experiments.

from yogurt on ARS milk agar and $CaCO_3$-MRS agar, Gram staining, and 16S rDNA sequencing were conducted.

To evaluate probiotic effect of yogurt fermented with *L. lactis* 11/19-B1, the silkworm infection model was used (Figure 4). Injection of *P. aeruginosa* to silkworm larva showed time-dependent and dose-dependent killing of silkworms. Silkworms were normally fed with a diet mixed with yogurt. Feeding of silkworms with a diet containing yogurt increased survival rate, resulting in about a 1,000-fold increase of LD_{50} for *P. aeruginosa* as compared to data in the absence of yogurt. To test if *L. lactis* 11/19-B1 has probiotic activity by itself, silkworms were fed a diet mixed with *L. lactis* 11/19-B1 viable cells (Figure 5). Feeding of silkworm with a diet containing *L. lactis* 11/19-B1 viable cells increased survival rate as well. Next, infection model with gram-positive pathogens were tested (Figure 6). Feeding of silkworms with a diet containing yogurt increased survival rate in methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) infection, resulting in about 2 and 4 fold increase of LD_{50} for MSSA and MRSA respectively. Yogurt feeding increased to a 2-fold survival rate in infection with *Enterococcus munditii*, causing flacherie disease in silkworms (*19*).

4. Discussion

4.1. *Screening LAB activating innate immunity as a yogurt starter*

In general, LAB has been thought to help human health in the gut as probiotics. Recently it has been noted that the activity of LAB stimulating the immune system is important (*14,15,20-23*). Yogurt is an example of fermented food with LAB. We studied to isolate LAB, which ferment milk to make yogurt and have an immune-stimulating activity as well. A few strains in many LAB spices ferment milk to form yogurt. In this study, we initially developed a new method to isolate milk-fermenting LAB.

To isolate LAB which grow and ferment milk, we developed a selection medium containing agar, milk as a nutrient, and ARS as a pH indicator, which show red in neutral conditions and yellow in acidic conditions. Because of its hydrophobicity and indiffusibility, ARS is useful to stain an acidic colony by fermentation. Since LAB has been known to colonize natural products including leaves and fruits of plants and gut in animals, we isolated LABs as starter candidates by screening acidic colonies on ARS agar from natural products.

As LAB belongs to Gram-positive bacteria, we

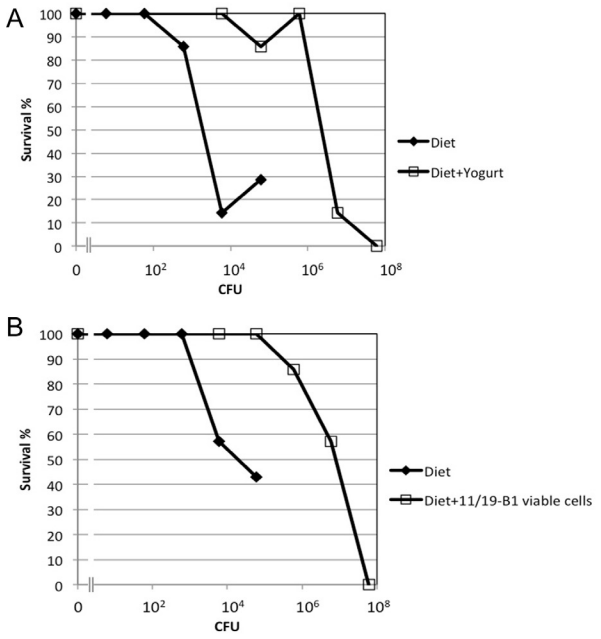

Figure 5. Probiotic effect of *L. lactis* 11/19-B1 yogurt on *P. aeruginosa* infection. Dose response of *P. aeruginosa* PAO1 on silkworm survival after 2 days. *P. aeruginosa* PAO1 was injected into 5th instar larva fed a diet with or without *L. lactis* 11/19-B1 yogurt **(A)** or viable cells (4×10^7 cfu/larva) **(B)**. Data represents a typical one of three experiments.

excluded Gram-negative bacteria isolated on ARS agar. We selected lactic-acid producing isolates on $CaCO_3$-MRS agar, in which lactic acid solubilizes $CaCO_3$ to form a clear spot around a colony. Then, we determined 16S rDNA sequences of each isolate. The level of 16S rDNA sequence similarity between *Lactococcus lactis* subsp. *lactis* and other *Lactococcus* species is 90-93% and it is 98-99% between *Lactococcus lactis* subsp. *lactis* and other subspecies of *Lactococcus lactis* (*24*). The identity of the 11/19-B1 strain as *L. lactis* was suggested by 99% similarity between the 11/19-B1 strain and *L. lactis* subsp. *lactis* IL1403 (*16*).

4.2. *Innate-immune activation in silkworm*

We determined the activity of LAB to stimulate innate immunity in silkworms using a muscle contraction assay (*3*). In this assay, insect cytokine PP upon innate immune activation induces muscle contraction in silkworms. Compared to a conventional method using macrophages, the muscle contraction assay does not require cell culture but is insensitive to LPS. Isolated LAB showed a variety of muscle contraction activity ranging from 0.3 to 105 U/mg. *L. lactis* 11/19-B1 had the highest activity. *L. lactis* 11/19-B1 fermented milk to form a yogurt with good flavor, which we used for further investigation.

4.3. *Acquisition of tolerance to bacterial infection by ingesting L. lactis 11-19-B1 yogurt*

We used silkworms as a surrogate animal to test

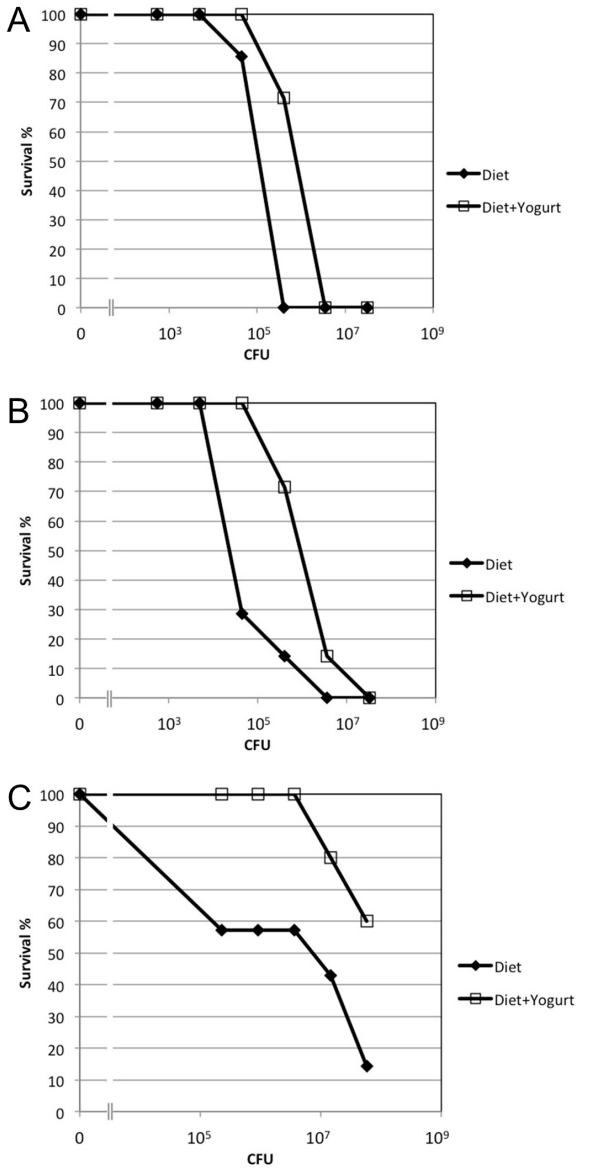

Figure 6. Probiotic effect of *L. lactis* 11/19-B1 yogurt on gram-positive pathogen infection. Dose response of gram-positive pathogens on silkworm survival after 2 days. Fifth instar larva fed a diet with or without *L. lactis* 11/19-B1 yogurt or viable cells . *S. aureus* MSSA1 **(A)**, MRSA4 **(B)**, or *E. mundtii* 12/5-1 **(C)** was injected into 5th instar larva fed a diet with or without *L. lactis* 11/19-B1 yogurt. Data represents a typical one of three experiments.

probiotic effect of yogurt. Silkworms were fed a diet containing yogurt without any problems. Silkworms fed yogurt had tolerance to lethality on infection with *P. aeruginosa*, *S. aureus*, and *E. munditii*. A thousand-fold decrease of LD_{50} of *P. aeruginosa* suggests that tolerance of yogurt-fed silkworm to *P. aeruginosa* was significant. Our previous results reported that silkworms acquire tolerance to *P. aeruginosa* infection by ingesting a diet mixed with the peptidoglucan of *P. aeruginosa* or *Lactobacillus plantarum* (*25*). In this study, we reported that an ingestion of viable cells or yogurt of *L. lactis* 11/19-B1 improved the survival of silkworms in *P. aeruginosa* infection. These data

suggest an innate-immune activation induced a primed immunity, an apparent acquired immunity, to microbial infection.

LAB would be expected to be an application for dairy products to help human health. Reports of a probiotic effect of LAB in an infection model has been limited. It was reported that *Bifidobacterium* protected *E. coli* O157 infection in germ-free mice (*26*). Oral administration of *Bifidobacterium longum* prevented *P. aeruginosa* gut-derived sepsis in a mouse model (*27*). Heat-killed *L. casei* protected against *P. aeruginosa* infection in mice (*28*). *L. lactis* is a nonpathogenic LAB known as not colonizing the mouth and gut, and not belonging to human gut flora (*29*). The *L. lactis* IL1403 genome was sequenced and a recombinant technique was established to construct a strain expressing specific antigens (*16,30,31*). Another recombinant expressing IL-10 was used to treat a mouse colitis model (*32-34*). Our data suggest that silkworms are a useful model animal to evaluate probiotic effects of LAB and isolated LAB would be expected to be an application for dairy products to help human health as well, even though a probiotic effect of isolated LAB on mammals including humans is unknown and needs further investigation.

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Original Article

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Acute oral toxicity test of chemical compounds in silkworms

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Summary **This study performed an acute oral toxicity test of 59 compounds in silkworms. These compounds are listed in OECD guidelines as standard substances for a cytotoxicity test, and median lethal dose (LD_{50}) werecalculated for each compound. Acute oral LD_{50} values in mammals are listed in OECD guidelines and acute oral LD_{50} values in silkworms were determined in this study. R^2 for the correlation between LD_{50} values in mammals and LD_{50} values in silkworms was 0.66. In addition, the acute oral toxicity test in silkworms was performed by two different facilities, and test results from the facilities were highly reproducible. These findings suggest that an acute oral toxicity test in silkworms is a useful way to evaluate the toxicity of compounds in mammals.**

Keywords: Acute oral toxicity test, animal model, silkworm

1. Introduction

Acute toxicity of chemical compounds can be evaluated by calculating LD_{50} values, *i.e.*, the dose that will kill 50% of animals in a test group. From the viewpoint of animal welfare, sacrificing a large number of mammals in an experiment raises ethical issues. At the end of 2002, the Organization for Economic Cooperation and Development (OECD) deleted TG 401, which is the method for evaluating the acute oral toxicity of compounds by calculating LD_{50} values in mammals (*1*). Given the issue of animal welfare, the fixed dose procedure (FDP; OECD 420), the acute toxic class method (ATC; OECD 423), and the up and down procedure (UDP; OECD 425) have been developed as alternatives to animal testing in TG 401. However, mammals must be sacrificed even when using these alternate methods. Cytotoxic tests of mammalian cells could predict the acute toxicity of compounds in mammal by measuring IC_{50} values (*2*). However, predicting the toxicity of compounds in mammals with those tests would be difficult since the absorption, distribution, and metabolism of compounds throughout the body and the excretion of compounds from the body are not taken into account. Techniques to predict the acute toxicity of compounds in mammals without sacrificing those animals must be devised to overcome these problems.

Silkworms have been bred over the long history of sericulture, and subjecting silkworms to a toxicity test poses fewer ethical problems. Moreover, techniques to rear a large number of genetically uniform individuals have been established. The current authors also recently reported that metabolic pathways in silkworms are similar to those in mammals and that the toxicity of some compounds to silkworms accords with their toxicity to mammals once weight is taken into account (*3*). Moreover, silkworms are large enough to inject a precise amount of a sample solution into either the hemolymph or the midgut (*4*). Hemolymph is akin to blood in humans and injection into the midgut is akin to oral administration in humans. In light of these facts, an acute oral toxicity test in silkworms might be alternative to testing mammals. Thus, the current study was conducted.

This study performed an acute oral toxicity test of 59 compounds in silkworms. These compounds are listed in OECD guidelines (*5*), and LD_{50} values

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were calculated for each compound. LD_{50} values in silkworms were calculated in this study and LD_{50} values in mammals are listed in OECD guidelines. LD_{50} values in silkworms and LD_{50} values in mammals were subjected to linear regression analysis. The acute oral toxicity test in silkworms was conducted two different facilities, and this study evaluated the between-laboratory reproducibility of their results.

2. Materials and Methods

2.1. *Acute oral toxicity tests in silkworms*

The toxicity of 59 compounds was evaluated in silkworms using an acute oral toxicity test. These compounds (Table 1) are listed as standard substances in OECD Guidelines. *Bombyx mori* eggs (Hu Yo × Tsukuba Ne or Kinshu × Showa) were purchased from Ehime Sansyu (Ehime, Japan) or Kougensha (Nagano, Japan) and raised as previously described (*6,7*). A two-fold dilution series containing each compound (50 μL) was injected into the midgut of fifth instar silkworm larvae with a disposable plastic syringe (Terumo, Tokyo, Japan). Silkworms were fed with Silkmate 2S (Nosan Co., Yokohama, Japan) every day and reared at 27°C. After 2 or 7 days, survival rates were measured and LD_{50} values were calculated based on the survival curve ($n = 7$).

Acute oral LD_{50} values in silkworms were determined at 2 days and acute oral LD_{50} values in mammals are listed in OECD guidelines. Linear regression analysis of the common logarithm of LD_{50} values in silkworms and LD_{50} values in mammals was performed to determine the correlation between these 2 values.

2.2. *Reproducibility of an acute oral toxicity test in silkworms conducted by two different facilities*

Two different facilities (Laboratory 1: Genome Pharmaceuticals Institute Co., Ltd., Tokyo, Japan, Laboratory 2: Noevir Co., Ltd. Tokyo Research Laboratory, Kanagawa, Japan) performed an acute oral toxicity test in silkworms, and LD_{50} values for 59 compounds were calculated after 2 days. Each facility calculated the LD_{50} values in silkworms, and the Pearson's product-moment correlation coefficient of the correlation between those 2 values was determined in order to examine the between-laboratory reproducibility of the toxicity test results.

3. Results

3.1. *Comparison of acute oral toxicity test results in silkworms and in mammals*

LD_{50} values in mammals and IC_{50} values in 3T3 cells and NHK cells are listed in OECD guidelines. Linear regression analysis of the LD_{50} values was performed, and the regression line and the determination coefficient were defined as below.

$\log (LD_{50}$ in mammals$) = 0.499 \times \log (IC_{50}$ 3T3 cells$) + 1.65 \quad (R^2 = 0.33)$

$\log (LD_{50}$ in mammals$) = 0.445 \times \log (IC_{50}$ NHK cells$) + 1.81 \quad (R^2 = 0.37)$

LD_{50} values for 59 compounds were calculated based on an acute oral toxicity test in silkworms (Table. 1). LD_{50} values in silkworms were calculated in this study and LD_{50} values in mammals are listed in OECD guidelines. Linear regression analysis of the 2 values was performed, and the regression line and the determination coefficient were defined as below.

$\log (LD_{50}$ in mammals$) = 0.860 \times \log (LD_{50}$ in silkworms$) + 0.168 \quad (R^2 = 0.66)$

Some of the 59 compounds produced LD_{50} values in silkworms that differed from LD_{50} values in mammals listed in OECD guidelines (Table 1). The LD_{50} values for physostigmine, nicotine, lindane, carbamazepine, propylparaben, and sodium hypochlorite were 10 or more times lower in silkworms than in mammals. The LD_{50} values for cycloheximide, sodium selenate, and phenylthiourea were 10 or more times higher in silkworms than in mammals.

3.2. *Reproducibility of an acute oral toxicity test in silkworms performed by two different facilities*

LD_{50} values in silkworms that are calculated by different facilities must be highly reproducible if an acute oral toxicity test in silkworms is to serve as an alternative to acute oral toxicity testing in mammals. Each facility calculated the LD_{50} values in silkworms, and the Pearson's product-moment correlation coefficient of the correlation between those 2 values was 0.88

4. Discussion

Cytotoxicity tests of compounds in mammalian cells or cell lines have been proposed as a way to predict the acute oral toxicity of compounds in mammals (*2*). IC_{50} values in 3T3 cells or NHK cells and LD_{50} values for 59 compounds in mammals are listed in OECD guidelines (*5*). Linear regression analysis of those IC_{50} values yielded an R^2 of 0.33 and linear regression analysis of LD_{50} values in mammals yielded an R^2 of 0.37. LD_{50} values that were calculated in the acute oral toxicity test in silkworms and LD_{50} values listed in OECD guidelines for mammals were subjected to linear regression analysis, which yielded an R^2 of 0.66 (Figure 1). These results suggest that, in contrast to a toxicity test in mammalian cells, an acute oral toxicity

Table 1. Acute oral LD_{50} values in silkworms calculated in this study

No.	Name	*Ref.* acute oral LD_{50} (*5*) (mg/Kg)	Laboratory 1		Laboratory 2	
			LD_{50} (Day 2) (mg/Kg)	LD_{50} (Day 7) (mg/Kg)	LD_{50} (Day 2) (mg/Kg)	LD_{50} (Day 7) (mg/Kg)
1	1,1,1-Trichloroethane	12,078	6,200	5,200	N.D.	1,700
2	2-Propanol	5,105	22,000	22,000	14,000	11,000
3	5-Aminosalicylic acid	3,429	460	160	N.D.	N.D.
4	Acetaminophen	2,163	1,700	870	2,300	1,800
5	Acetonitrile	3,598	43,000	36,000	25,000	20,000
6	Acetylsalicylic acid	1,506	1,400	1,400	1,900	1,500
7	Arsenic III trioxide	25	100	100	16	16
8	Atropine sulfate	819	3,400	1,100	N.D.	1,300
9	Boric acid	3,426	3,100	1,600	1,300	830
10	Busulfan	12	42	42	N.D.	N.D.
11	Cadmium II chloride	135	57	51	140	87
12	Caffeine	310	560	290	800	300
13	Carbamazepine	2,805	280	120	N.D.	210
14	Carbon tetrachloride	3,783	3,400	2,400	1,200	1,200
15	Chloral hydrate	638	1,700	990	1,600	1,580
16	Citric acid	5,929	10,000	9,500	6,500	6,500
17	Colchicine	15	23	1.8	N.D.	5.4
18	Cupric sulfate $5H_2O$	474	650	460	940	610
19	Cycloheximide	2	1,875	1,875	2,300	2,100
20	Dibutyl phthalate	8,892	45,000	30,000	N.D.	18,000
21	Dichlorvos	59	310	15	0.42	0.42
22	Diethyl phthalate	9,311	3,800	3,500	4,400	4,000
23	Digoxin	28	96	6.1	24	17
24	Dimethylformamide	5,309	20,000	18,000	25,000	16,000
25	Ethanol	11,324	56,000	34,000	28,000	25,000
26	Ethylene glycol	7,161	33,000	41,000	46,000	46,000
27	Gibberellic acid	6,040	7,100	4.4	N.D.	N.D.
28	Glycerol	19,770	35,000	35,000	54,000	54,000
29	Haloperidol	330	1,100	69	N.D.	N.D.
30	Hexachlorophene	82	20	12	67	59
31	Lactic acid	3,639	36,000	30,000	5,100	4,500
32	Lithium I carbonate	590	220	5.4	N.D.	N.D.
33	Mercury II chloride	40	120	50	58	40
34	Methanol	8,710	6,900	3,900	8,300	8,000
35	Nicotine	70	15	3.3	3.6	3.7
36	Phenol	548	860	790	1,000	940
37	Phenylthiourea	3	320	170	240	240
38	Potassium Cyanide	7	25	25	74	82
39	Potassium I chloride	2,799	6,800	5,800	10,000	7,900
40	Procainamide	1,950	7,000	4,100	N.D.	5,900
41	Propranolol HCL	466	570	540	540	490
42	Propylparaben	6,332 (mouse)	670	370	640	580
43	Sodium chloride	4,046	6,500	5,000	6,800	4,900
44	Sodium Dichromate Dihydrate	51	170	170	610	530
45	Sodium hypochlorite	10,328	1,500	980	830	650
46	Sodium I fluoride	127	110	70	110	96
47	Sodium oxalate	633	1,100	1,100	1,300	1,300
48	Sodium selenate	3	50	39	150	110
49	Thallium I sulfate	25	92	10	1,100	1,100
50	Trichloroacetic acid	5,229	5,200	3,500	1,500	1,200
51	Valproic acid	995	3,500	3,500	7,900	6,100
52	Verapamil HCL	111	490	370	1,200	1,100
53	Xylene	4,667	2,200	2,200	2,100	1,100
54	Amitriptyline HCL	348	390	210	340	290
55	Chloramphenicol	3,491	1,100	980	350	320
56	Epinephrine Bitartrate	4 (mouse)	14	14	N.D.	N.D.
57	Lindane	100	27	12	2.3	2.3
58	Physostigmine	5	0.76	0.46	0.75	0.65
59	Strychnine	6	44	4.1	N.D.	N.D.

"*Ref.* acute oral LD_{50}" means LD_{50} values for adult laboratory rats according to the literature (*5*) unless otherwise specified.

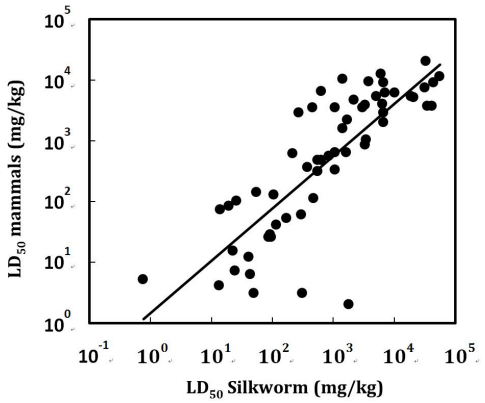

Figure 1. Linear regression analysis of acute oral LD_{50} values for 59 compounds (listed in OECD guidelines) in silkworms and in mammals. Acute oral LD_{50} values for 59 compounds were calculated using the data on silkworm survival after 2 days. Acute oral LD_{50} values in mammals were obtained from the literature. The common logarithm of LD_{50} values in silkworms and in mammals was determined and regression analysis of these 2 values was performed. The regression line was defined as: log (LD_{50} in mammals) = 0.860 × log (LD_{50} in silkworms) + 0.168 (R^2 = 0.66)

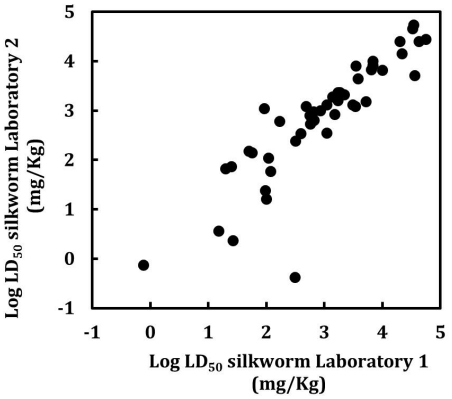

Figure 2. Comparison of acute oral LD_{50} values in silkworms calculated by two different facilities. An acute oral toxicity test in silkworms was performed by two different facilities (Laboratories 1 and 2). Acute oral LD_{50} values for 59 compounds (LD_{50} in silkworms: mg/Kg) after 2 days were calculated by each facility. Each facility calculated the LD_{50} values in silkworms, and the Pearson's product-moment correlation coefficient of the correlation between those 2 values was 0.88. Laboratory 1: Genome Pharmaceutical Institute Co., Ltd. Laboratory 2: Noevir Co., Ltd. Tokyo Research Laboratory.

test in silkworms yields results that are more closely in line with the results of an acute oral toxicity test in mammals. A previous study by the current authors reported that some compounds are metabolized in silkworms in the same manner as in mice (*3*). An *in vitro* cytotoxicity test cannot reflect the absorption, distribution, and metabolism of compounds throughout the body and the excretion of compounds from the body, and this leads to disparities in the oral acute toxicity and cytotoxicity of some compounds. These problems might be overcome through the use of silkworms and more consistent results with mammalian test could be obtained rather than cytotoxicity test.

In this study, the LD_{50} values for some compounds were 10 or more times lower in silkworms than in mammals or 10 or more times higher in silkworms than in mammals. Compounds with LD_{50} values that were 10 or more times lower in silkworms were physostigmine, nicotine, lindane, carbamazepine, propyl paraben, and sodium hypochlorite. Compounds with LD_{50} values that were 10 or more times higher in silkworms were cycloheximide, sodium selenite, and phenyl thiourea. Physostigmine is a carbamate insecticide and dichlorvos has been used as an organic phosphorus-based insecticide; physostigmine and dichlorvos are known to inhibit acetylcholinesterase (*8,9*). Nicotine, an acetylcholine receptor agonist, has been used as an insecticide (*10*). Lindane is an insecticide that is known to be a GABA receptor antagonist (*11*). Therefore, silkworms may be more sensitive to these compounds than mammals. Cycloheximide was one compound with an LD_{50} value that was 10 or more times higher in silkworms than in mammals. Cycloheximide may be hydrolyzed in the midgut of silkworms because of the alkaline environment there (pH 9-11).

This study evaluated the reproducibility of an acute oral toxicity test in silkworms by having two different facilities perform the test. Each facility calculated LD_{50} values in silkworms, and the Pearson's product-moment correlation coefficient of the correlation between those 2 values was 0.88 (Figure 2). Therefore, the acute oral toxicity test in silkworms has a high level of between-laboratory reproducibility. The two facilities found that LD_{50} values for some compounds, such as thallium sulfate and dichlorvos, differed by 10 times or more in silkworms. In the acute oral toxicity test, each sample was directly injected into the midgut of silkworms. Therefore, this discrepancy in LD_{50} values may be due to how adeptly the sample was injected.

Results of this study indicated that, in contrast to a cytotoxicity test using mammalian cells, an acute oral toxicity in silkworms yields results that are more closely in line with the results of an acute oral toxicity test in mammals. Furthermore, the current results suggest that an acute oral toxicity test in silkworms has a high level of between-laboratory reproducibility. Therefore, an acute oral toxicity test in silkworms is a useful alternative to testing the acute oral toxicity of chemical compounds in mammals.

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