

## Review

### Development and implementation of silkworm cell-free protein synthesis systems

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**We have developed a cell-free protein synthesis (CFPS) technique that makes use of an extract prepared from the organs of the silkworm—an organism renowned in synthetic biology as a “protein manufacturing plant.” This extract, prepared from the posterior silk gland of silkworm larvae, has enabled us to develop a stable, high-yield, and exceedingly implementable CFPS pipeline: a silkworm CFPS system. By demonstrating its capability to efficiently produce a variety of disease-associated proteins, we have demonstrated the significant utility of this system in drug development research. In an effort to make our technology widely available to biological and pharmacological researchers, we have partnered with Ozeki Corporation to launch a venture corporation that leverages our silkworm CFPS system to provide contract-based protein production solutions.**

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**Key words:** Cell-free protein synthesis system, silkworm, *Bombyx mori*, posterior silk gland.

#### Introduction

The first successful sequencing of a human genome in 2003—now nearly 17 years ago—paved the way for meteoric advancements in genomic analysis solutions, which have together ushered in an age in which the entire genome of nearly any organism can be quickly and accurately decoded. In light of these developments, the functional analysis of proteins—gene products, whose production is the primary molecular task of life itself—has become an increasingly critical area of research (e.g., protein-protein interaction

analysis). Drug development, which seeks to establish compounds that can bind to and regulate protein activity (lead compounds, also known as developmental candidates), has also become an active area of pharmacological research. However, these endeavors all first require the production, by some means, of target proteins.

Nowadays, target proteins are most commonly produced via expression systems in genetically modified organisms (*E. coli*, yeast, and cultured insect and mammalian cells). Very recently, however, cell-free protein synthesis (CFPS) systems, where target proteins are synthesized *in vitro*, have entered the spotlight, due to their simplicity and convenience. In CFPS systems, protein synthesis reactants, such as substrates and energy sources, are added to a cell extract

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containing the machinery necessary for protein synthesis (ribosomes, elongation factors, etc.), thereby assembling an *in vitro* genetic translation system, which can use the mRNA that codes for the target protein as a template to synthesize the protein itself (Fig. 1). At times DNA is used instead of mRNA, and transcription and translation are carried out side-by-side. Existing CFPS systems require expensive reagents and are not suitable for protein production at an industrial scale. However, in comparison to expression systems in living cells, CFPS systems, which do not use living cells, i) are not dependent on the various factors required to keep cells alive, ii) can generally run reactions from start to finish in a short amount of time, iii) can handle multi-specimen processing, iv) can produce cytotoxic proteins, v) can easily accommodate unnatural amino acids, facilitating the labeling of target proteins, and vi) are not subject to the regulations governing genetic modification experiments. These advantages in comparison to systems involving living cells make CFPS systems a remarkably useful tool for the production of target proteins, at the laboratory scale, for functional analysis.

CFPS research predates genetic modification technology. In the 1950s, it was discovered that crushed-cell extracts retained their ability to perform protein synthesis; since then, research has been conducted on extracts prepared from *E. coli*, wheat germ, rabbit reticulocytes, cultured insect cells, and cultured human cells [1]. Reagent kits containing these extracts have been developed and are currently being sold by several biotechnological companies. In recent years, research into this technology, primarily in Japan, has led to the development of highly efficient expression systems capable of outputting several milligrams of protein per mL of reaction solution through the use of *E. coli*- and wheat germ-derived extracts [2, 3]. High-throughput synthesis pipelines, which can produce hundreds of protein types overnight, and robots that automate this process have been developed. Nevertheless, CFPS systems have yet to become mainstream protein production tools.

Amidst these advancements, our group achieved the production of a variety of proteins using a live-cell silkworm-baculovirus expression system.

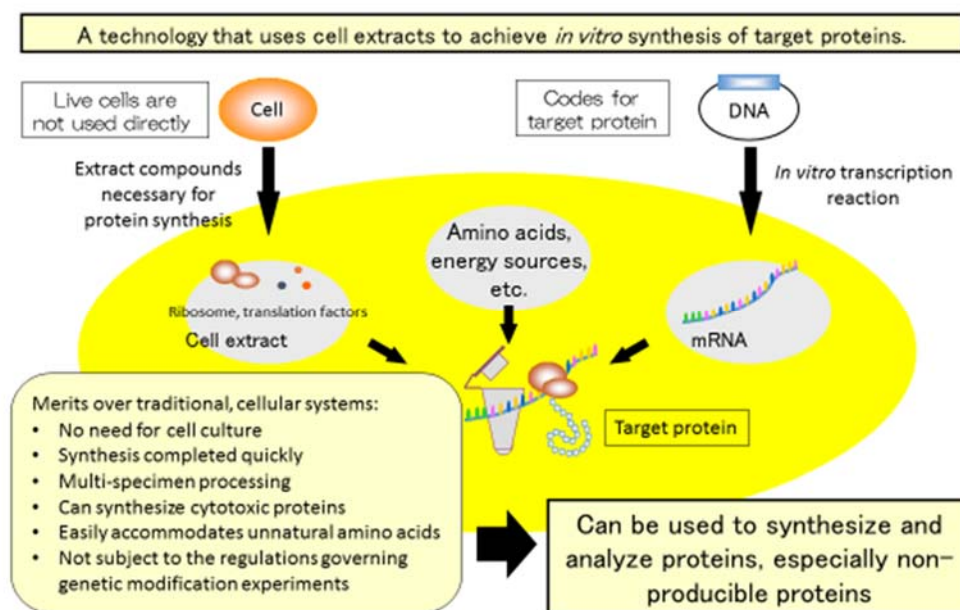
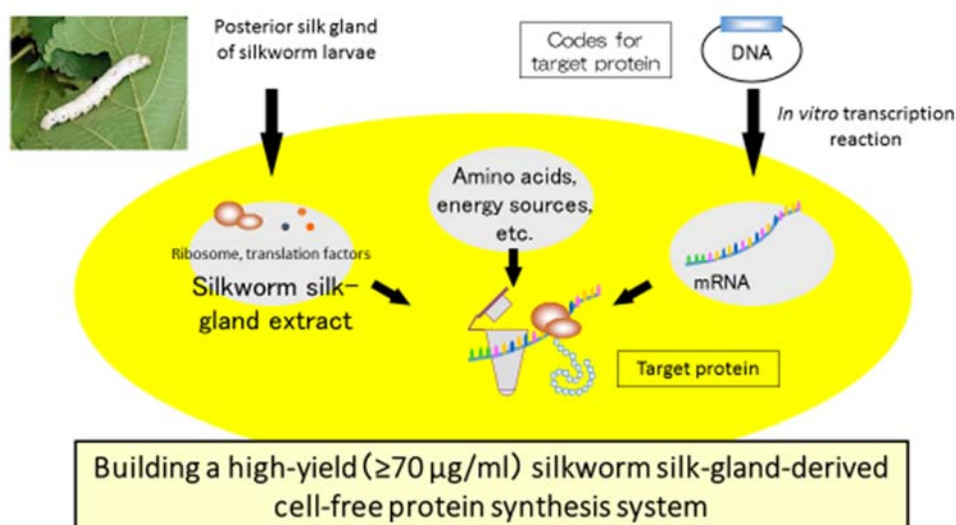


Fig. 1. Outline of CFPS systems.



**Fig. 2. Silkworm CFPS systems.**

Further, by focusing on the silkworm itself—an organism known to be a “protein production factory”—we have pioneered a CFPS system that uses an extract prepared from silkworm organs [4]. We have also developed a stable, high-yield, implementable CFPS system (our silkworm CFPS system) using extracts prepared from the posterior silk glands of silkworm larvae (Fig. 2). By demonstrating its capability to efficiently produce a variety of disease-associated proteins, we have established the significant utility of this system in drug development research. Finally, in an effort to make our technology widely available to biological and pharmacological researchers, we have partnered with Ozeki Corporation to launch a venture corporation that leverages our silkworm CFPS system to provide contract-based protein production solutions. In this manuscript, we present the process by which this technology was researched, developed, and made implementable, and discuss the future perspectives of silkworm CFPS systems.

#### **Basic research on silkworm CFPS systems**

Our basic research on silkworm CFPS systems was performed between January 2001 and March 2004 at the Rengo Co. Central Research Institute, in collaboration with Dr. Sumiharu Nagaoka of the Kyoto Institute of Technology Graduate School of Science and Technology. Silkworms (*Bombyx mori*) were chosen as the insect from which to produce the extracts due in part to its large size, which allows for easy rearing and organ extraction. Trials of cell-free translation in silkworms were already underway as of the 1970s [5]. Using an extract prepared from the posterior silk gland (the organ that produces the fibroin protein in silk) of 5- to 6-day-old fifth-instar silkworm larvae and endogenous mRNA as a template, these researchers succeeded in the *in vitro* synthesis of silk fibroin protein. However, their synthetic yield was quite low, and the experiment did not attempt synthesis involving exogenous mRNA; thus, their system was not a truly implementable protein synthesis system. Using commercial luciferase mRNA as our template, we investigated the ability of systems constructed using extracts of silkworm

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organs and structures (silkworm larvae middle and posterior silk glands and fat pads, embryos (eggs), and pupae) to synthesize luciferase protein. We observed luciferase synthesis when using extracts containing posterior silk glands of fifth-instar 2- to 7-day-old silkworm larvae, their fat pads, and silkworm embryos (that had been kept warm for 2 days). Among these, a system using an extract prepared from the posterior silk glands of fifth-instar 4-day-old silkworm larvae exhibited the highest yield. Around the fourth day of its fifth instar, the posterior silk gland of silkworm larvae produced fibroin, the primary structural component of silkworm cocoons, at peak capacity. Thus, we hypothesized that the application of this high synthetic capacity to our CFPS system would result in high yields. Therefore, we chose to target the posterior silk glands of fifth-instar 4-day-old silkworm larvae, and carried out the following manipulations to improve our yields further: i) refinement of extraction procedure: glycerol was added during extraction, which brought the soluble fibroin present in our extracts out of solution and dramatically increased yields; ii) optimization of reaction compositions: the optimal amount of each reaction ingredient to be added to the reaction mixture was determined; iii) use of translation promoter sequences: the effects of the insertion of major insect protein genes or baculovirus polyhedrin 5' UTR upstream of the start codon on the template expression on protein yield were investigated and the sequence that promoted translation was identified. After applying what we learned from these manipulations, we were able to synthesize approximately 30 µg of luciferase from 1 mL of reaction solution, which is nearly 10 times as much as that produced using traditional commercial rabbit reticulocyte extract CFPS systems. We were also able to efficiently synthesize other proteins. Thus, we achieved our developmental concept: to build an animal-origin,

high-yield, optically analyzable (i.e., colorless and transparent reaction solution) protein synthesis system. However, the preparation of larval posterior silk gland involves several delicate steps, including the dissection of silkworm larva, the extraction of their organs, and washing, which result in a process that is long and laborious as a whole. Further, the yields of separate lots of prepared extract differed, preventing us from maintaining a consistent level of quality. For these reasons, we determined that, at the time, the stable, low-cost production of silkworm larvae posterior silk gland extract was unrealistic, and our research and development of silkworm CFPS systems was put on hiatus. In the meantime, we made use of the knowhow gained from our work with the silkworm CFPS systems to develop a new CFPS system that used an extract prepared from cultured insect cells, which can be consistently produced at scale [6]. After transferring to Shimadzu Corporation, this new technology was commercialized into the cultured-insect-cell-derived CFPS reagent kit Transdirect *insect cell*, which launched in December 2004 and continues to be marketed to this day [6].

### **Research on the implementation of silkworm CFPS systems**

In April 2008, I joined the National Institute of Technology, Okinawa College, where my group and I resumed development of stable, high-yield silkworm CFPS systems by focusing on the reliable rearing of silkworms, the simplification of silk gland extraction techniques, and solutions for extract preparation, among other areas. This work is outlined in the sections below.

#### **(1) Investigating translation promoter sequences**

In an effort to further improve yields, we screened for translation promoter sequences. In addition, to identify the portions of the selected

sequences that directly promoted translation (i.e. the functional sequences), we evaluated the effects of shortening translation promoter sequences on the protein yield. We constructed a total of 6 types of  $\beta$ -galactosidase expression plasmids containing the polyhedrin gene from nuclear polyhedrosis viruses in *Malacosoma neustria*, *Autographa californica*, *Ectropis oblique*, *Choristoneura rosaceana*, and *Spodoptera frugiperda* and the 5'-UTR of the rabbit reticulocyte  $\beta$ -globin gene. PCR was used to prepare linear DNA, which was then used to synthesize mRNA for use as a template for the synthesis of  $\beta$ -galactosidase in our silkworm cell-free system. Yields were calculated by examining the activity of our synthesis product solution in reference to the specific activity of  $\beta$ -galactosidase. We found that the 40-base-long 5'-UTR of the polyhedrin gene in *Spodoptera frugiperda* nuclear polyhedrosis virus promoted translation best. Next, we successively shortened this sequence in 2-nucleotide increments from either the 5'- or 3'-end; a total of 38 plasmid types were constructed containing these sequence variants, and their relative effect on the synthesis yields of  $\beta$ -galactosidase was examined. We found that the sequence by 18 nucleotides from the 3'-end, to [5'-ACATTGTGAAAAAATAAA-3'], produced approximately 1.3 times as much as the yield obtained with the original sequence. Therefore, this sequence was determined to be a functional promoter sequence and was used in our system to synthesize the target proteins more efficiently.

## **(2) Development of middle silk gland CFPS systems**

Another line of research we undertook examined the use of middle silk glands—which are larger and easier to extract—in our silkworm CFPS systems. We explored methods for the preparation of middle silk gland extract and the development of CFPS systems that employed this extract. Although the

middle silk gland produces sericin (another component of silk cocoons), sericin production is accompanied by the production of fibroin (the primary component of silk cocoons) in the posterior silk gland in wild-type silkworms. Since the middle silk gland is quite viscoelastic, it is not suitable for the preparation of CFPS extracts. Thus, we crossed wild-type silkworms with the mutant Sericin Hope line, which specifically does not produce fibroin, to obtain middle silk glands from hybrid species [7]. An incision was made in the intersegmental membrane between the compound legs on the 3rd and 4th segments of this hybrid silkworm larvae using scissors, such that the middle silk gland could be easily removed using tweezers. This method allowed us to excise the middle silk glands in 1/10th the time previously taken to excise posterior silk glands, thereby significantly reducing the time taken to prepare the cellular extracts. Approximately 30  $\mu\text{g/ml}$  of  $\beta$ -galactosidase was synthesized when using extract prepared from fifth-instar, 2- to 3-day-old middle silk glands. These results demonstrated that we successfully developed a CFPS system involving a very simple extract preparation method [8, 9].

## **(3) Improvement of posterior silk gland CFPS systems**

The posterior silk gland of fifth-instar silkworm larvae is tasked with producing large amounts of fibroin in a relatively short time and possesses an exceedingly high protein synthetic capacity. We used this information in our aim of improving our posterior silk gland silkworm CFPS system. First, we worked to develop a reliable method of silkworm rearing using artificial feed. Next, we developed a method to extract the posterior silk glands of fifth-instar 4-day-old silkworm larvae easily and quickly, in a maximum of 20 seconds. In addition, in order to preserve their high synthetic capacity, we developed a simple, mild, temperature-controlled preparation method for

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large amounts of gland extract. Finally, we applied the translation promoter sequence we had developed in our earlier experiments. Using this new, mass-prepared extract, we succeeded in reliably synthesizing  $\beta$ -galactosidase at a yield of 70  $\mu\text{g/ml}$  or more. The development of these techniques allowed us to establish a maximum-yield, highly implementable CFPS system that uses animal-derived extracts from silkworms, which can be cheaply procured and raised.

### (4) Development of Eri silkworm CFPS systems

The silkworm moth-based CFPS system poses some technical challenges, namely the fact that the posterior silk gland in these species is small and difficult to excise, and that middle silk glands do not have the synthetic capacity to produce high yields. In an effort to address these drawbacks, we investigated the Eri silkworm, belonging to the family Saturniidae. The silk gland of Eri silkworms develops rapidly in their fifth instar, and the species' middle and posterior silk glands are nearly identical. Moreover, both can be quite easily excised. In an effort to use these easily extracted silk glands and improve our yield to implementable levels, we tested various preparation conditions. When the extract was prepared using a mix of extraction solvent and silk glands excised from fifth-instar 2- to 3-day-old Eri silkworm larvae in a 5:4 weight ratio, 4.2  $\mu\text{g/ml}$  of  $\beta$ -galactosidase was synthesized. Thus, we were able to develop an Eri silkworm CFPS system that preserved our workability improvements, albeit with a lower yield than the silkworm posterior and middle silk gland extract systems [10].

### (5) Selecting implementable CFPS systems

In an effort to improve the yield of CFPS systems using normal silkworm larvae posterior silk gland extracts, we tested various extract preparation conditions, optimized the composition of the reaction solution, and applied translation promoter sequences. Through these iterations, we

succeeded in building a system whose yield (70  $\mu\text{g/ml}$ ) was over double that of our initial efforts (30  $\mu\text{g/ml}$ ). In terms of the ease of extract preparation, the total volume of extract obtainable from one silkworm in the posterior silk gland system was superior to that from both the middle silk gland and Eri silkworm systems. We therefore chose the silkworm larvae posterior silk gland CFPS systems for industrialization.

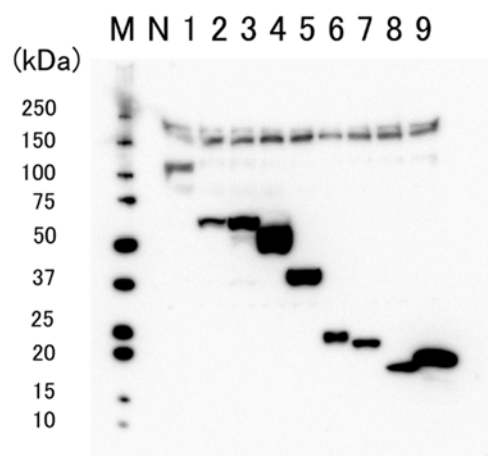
### Production of disease-associated proteins

For proteins synthesized using the silkworm CFPS systems to be used in drug development research, simple and convenient methods for their purification must also be developed. To this end, we constructed a total of 15 types of tagged expression plasmids, including GST tag, *Strep*-tag II, His tag, FLAG tag, HA tag, and His-GST tag. These plasmids were then synthesized and the results compared. We found that the *Strep*-tag II sequence was most suitable for tag purification, yielding a high purity product ( $\geq 90\%$ ), at a low cost (approximately ¥2000 per synthesis), available patent-free. With this method, we were able to design a silkworm CFPS system pipeline for the rapid production and purification of disease-associated proteins. Next, we created a database containing all the disease-associated proteins important in drug development research. We used this database to select several disease-associated proteins for synthesis from a total of 646 protein targets of medications approved by the American Food and Drug Administration (FDA) based on the following criteria: i) proteins often used in the developmental pipelines of pharmaceutical companies; ii) proteins that have received increased attention in the literature in the last 10 years; iii) excluded proteins, such as G-protein coupled receptors, transporters, ion channels, nuclear receptors, and other membrane proteins, that are difficult to synthesize. On the

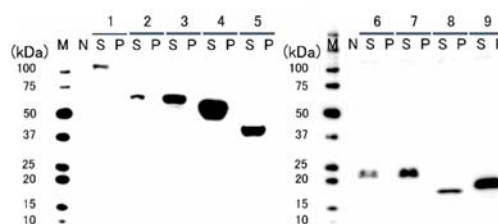
basis of these criteria, we selected 9 disease-associated proteins we expected to be in high demand (e.g. Janus kinase-2, matrix metalloproteinase 2, prostaglandin-endoperoxide synthase 2, SRC proto-oncogene non-receptor tyrosine kinase, mitogen-activated protein kinase 1, vascular endothelial growth factor A, interleukin 6, tumor necrosis factor, and interleukin 1-beta) (Table 1). We synthesized the genes of these 9 proteins externally, then inserted them into expression vectors containing the *Strep*-tag II sequence, and created expression plasmids for each disease-associated protein. These were used as templates for mRNA synthesis, followed by silkworm CFPS. Western blot analysis of the synthesis reaction solutions clearly indicated that all 9 disease-associated proteins were successfully synthesized in soluble form (Figs. 3 and 4). In terms of tag purification, interleukin 1-beta and mitogen-activated protein kinase 1 were found to be easily purified, forming a single coherent band on an SDS-PAGE gel after just one run through a purification column. This clearly indicates that disease-associated proteins used in drug development research can be efficiently synthesized in soluble form in silkworm CFPS systems.

**Table 1. Selected human disease-associated proteins.**

Protein name (abbreviation)	Weight (kDa)
Janus kinase 2 (JAK2)	130
Matrix metalloproteinase 2 (MMP2)	74
Prostaglandin-endoperoxide synthase 2 (PTGS2)	67
SRC proto-oncogene, non-receptor tyrosine kinase (SRC)	60
Mitogen-activated protein kinase 1 (MAPK1)	41
Vascular endothelial growth factor A (VEGFA)	27
Interleukin 6 (IL6)	21
Tumor necrosis factor (TNF)	17
Interleukin 1 beta (IL1B)	17



**Fig. 3. Western blot analysis of synthesis products. M: Molecular weight marker, N: negative control (-mRNA reaction solution), 1: JAK2, 2: MMP2, 3: PTGS, 4: SRC, 5: MAPK1, 6: VEGFA, 7: IL6, 8: TNF, 9: IL1B.**



**Fig. 4. Solubility analysis of synthesis products. S: supernatant fraction, P: precipitate fraction, other labels are the same as in Fig. 3.**

**Launching a venture corporation that applies these research results**

In an effort to further refine and implement our newly developed technology, we founded the NIT Okinawa venture corporation Silk Renaissance Co., Ltd. in Uruma, Okinawa, on January 26, 2018. Silk Renaissance operates in two primary arenas: i) supporting drug development via the application of silkworm CFPS technology and ii) supporting regenerative medicine via the application of polymeric sericin [11], discovered through silkworm CFPS research. For more details, please

visit the Silk Renaissance website (<https://www.silk-r.jp/>). We hope that these efforts will help to shape Okinawa into a hub of drug development research. Below, I will briefly detail some of the work Silk Renaissance has done.

### **(1) Investors**

Securing funding is a critically important part of the start-up phase of any venture corporation. Silk Renaissance applied for and received funding from three start-up support initiatives: i) Venture Business Startup Support Project 2017-19, by the Okinawa Industry Promotion Public Corporation; ii) OKINAWA Startup Program 2018-2019, by the Bank of the Ryukyus Ltd. and Okinawa Times Co., Ltd.; iii) FY 2018-2019 Intellectual/Industrial Cluster Support Network Strengthening Project (for research seed commercialization/human resource development support), by the Okinawa Center for Science and Technology Promotion. These coordinators provided fund-matching services and proactive, hands-on support in the creation of detailed business plans. As a result, we successfully secured a total of ¥60 million through co-financing from the Bank of the Ryukyus' Sixth Industrialization Fund, the BOR Venture Fund, and the Okinawa Development Finance Corporation, as well as through a capital loan from the Okinawa Development Finance Corporation. This capital allowed us to commence business activity.

### **(2) Business partnerships**

Alongside securing investors and funding, we have worked to expand our business by forming business partnerships. Soon after starting our business, Silk Renaissance entered into a use license agreement regarding silkworm CFPS with Ozeki Corporation, which is developing contract-based protein production solutions using yeast and genetically modified silkworms. This agreement allows both Silk Renaissance and Ozeki to develop services using our technologies, as well as provide multifaceted services to the market from different

vendors. On June 19, 2019, a press release was issued by the Okinawa Prefectural Office congratulating Silk Renaissance and Ozeki for being the first companies to launch a CFPS service using silkworm posterior silk gland extract, declaring that Ozeki would be the first to officially begin offering this service. This service is unique for several reasons, including: i) a rapid protein synthesis that allows for the synthesis and delivery of target proteins in a very short time (15 business days); ii) high synthetic yields ( $\geq 70$   $\mu\text{g/ml}$ ); iii) capable of achieving the high-fidelity synthesis of proteins from humans and other mammals in soluble form. For more details on this service, please visit Ozeki's website ([https://www.ozeki.co.jp/food\\_bio/protein.html](https://www.ozeki.co.jp/food_bio/protein.html)).

### **Conclusion**

As the founders of Silk Renaissance, a venture corporation providing silkworm CFPS system technology, we have worked to develop services to support the fields of drug development and regenerative medicine, and thereby built our business foundation. We aim to apply our ability to rapidly synthesize target proteins to the fullest, and within the next 10 years, hope to work with pharmaceutical companies that wish to make great strides in drug development through the development of protein therapeutics. For example, we hope to apply cell-free system manufacturing techniques to the production of emergency-measure vaccines for healthcare workers in the event of future pandemic breakouts of infectious diseases, such as highly pathogenic influenza. Through these efforts, we hope to realize the revitalization of the sericulture industry: a silk renaissance.

### **Acknowledgements**

The research and development detailed here were carried out in various places and at various



times. Basic research on silkworm CFPS systems was carried out at the Rengo Co., Ltd. Central Research Institute between January 2001 and March 2004. Research on the implementation of silkworm CFPS systems was carried out from April 2008 to the present at the National Institute of Technology, Okinawa College. This paper was written on behalf of the many researchers who have participated in the research efforts. I would like to take this opportunity to thank these individuals.

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