JARQ 38 (4), 235 – 240 (2004) http://www.jircas.affrc.go.jp

REVIEW

Development of an Automatic Silkworm Larvae Hemolymph Collection System by Infrared Laser Beam Incision Technique

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Abstract

To establish an insect factory for mass production of useful substances using silkworms, the author developed a silkworm hemolymph collection system to efficiently collect hemolymph from 5th-instar larvae of silkworms, *Bombyx mori*. The system automatically performs the serial processes of feeding silkworm larvae to the system, incision, collection of hemolymph, and removal of silkworms after hemolymph collection. This system consists primarily of a silkworm feeder, a conveyer chain with holders for the transport of silkworms, a CO_2 laser for infrared laser beam incision of silkworms, a container for hemolymph collection, and two coolers. The hemolymph collection from 4 and 5-day 5th-instar silkworm larvae anesthetized at low temperature could be collected continuously in less than 2.5 min per larva, giving a good prospect for a one-man operation. The amount of hemolymph was 12–13% of the silkworm larva body weight.

Discipline: Biotechnology
Additional key words: insect factory, baculovirus, NPV

Introduction

Maeda et al.[4] developed a technique to utilize the high protein synthesizing ability of silkworms for the production of useful substances such as medical and animal drugs, and agricultural chemicals by inoculating recombinant baculovirus (BmNPV) in 5th-instar silkworms. They demonstrated the possibility of producing human interferon by this method. Vaccines for pets produced by this method have become commercially available[6]. This new production method, in which the living body of an insect, typically the silkworm, is used as a factory that produces useful substances, is called an "insect factory"[2]. Presently, a system for efficient mass production of useful substances in an insect factory with a scale of 10–20 thousand silkworms has not been realized. The construction of a system in which various manufacturing processes are automated is expected. However, the most difficult process of systematization of these processes is the automation of the collection of hemolymph that contains the useful substances from the bodies of the silkworms reared for 4–5 days after recombinant baculovirus inoculation. Therefore, as part of the research for the establishment of an innovative insect factory, the author previously developed a method to efficiently collect hemolymph by infrared laser beam incision (CO_2 laser) of only the integument of 4 and 5-day 5th instar silkworm larvae anesthetized at 0ºC for 20 min to mechanize the process of hemolymph collection, for replacing the conventional procedure[3].

By incorporating the above new hemolymph collection method, the author has further developed a silkworm hemolymph collection system that automatically performs the series of hemolymph collection processes from feeding silkworms anesthetized at low temperature into the system, making an incision, collecting hemolymph, and removing the silkworms from holders

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Received 30 March 2004; accepted 8 June 2004.

after hemolymph collection. This report is an outline of this system.

Automatic silkworm larvae hemolymph collection system

In the newly developed system (Fig. 1), various operational units (each process) for the collection of hemolymph from silkworms anesthetized at low temperature were arranged along a single conveyer chain that circulated around the system. Table 1 shows the specifications of this system.

The system was composed of a feeding unit supplying 4 and 5-day 5th-instar silkworm larvae; a transport unit consisting of a conveyer chain (main conveyer) that can be driven either forward or backward at variable speed and silkworm holders which were parallel pairs of plate springs attached to the main conveyer at intervals of 64 mm; an incision unit consisting of a CO_2 laser with a scanner generating a maximum output of 12 W and a sensor for timing of incision (beginning of laser irradiation); a hemolymph collection unit consisting of a gutter-like container that collected hemolymph after incision and 2 coolers that kept the container cool; and a removal unit that released the silkworms from the holders and recovered them after hemolymph collection. The feeder unit of the system consisted of a feeding chute, a conveyer chain with transfer arms (sub-conveyer; having the same specifications as the main conveyer), holders serving as jigs to receive silkworms, a feeding timer buzzer, an electromagnetic clutch, and universal joints. This unit was placed on the right side of the CO_2 laser (Fig. 2). The feeding unit was driven by connecting it with the motor of the hemolymph collection system via universal joints. The difference in the position of silkworm transfer was eliminated by driving the two conveyer chains at the same speed.

To prevent deterioration of the collected hemolymph due to melanosis or other biochemical causes, a 0.88% physiologic saline solution supplemented with 5 mg phenylthiourea was added in the hemolymph collecting container to a depth of about 5 mm in advance. The temperature of the collected hemolymph was maintained at 5ºC using the 2 coolers.

Methods

The performance of the newly developed system

Table 1. Specification of developed hemolymph collection system for silkworm

Feeding unit	Silkworm chute:	An inclined structure with the shape of half a funnel through which silkworms are fed into the transfer arm Equipped with a 0.1 MPa compressor and air nozzle that blows assist air into the chute
	Conveyer chain (sub):	Pitch 15.87 mm, link number 114, length 1,778 mm
	Transport speed:	0–85 mm/s; driven by the motor of the transport unit via universal joints
	Transfer arm:	Moved 22 mm up and down by a cam Pick up and transfer of silkworms
Transport unit	Conveyer chain (main):	Pitch 15.87 mm, link number 512, length 8,125 mm
	Holders:	Pairs of plate springs that are opened and closed by a cam to hold silkworms
	Transport speed:	0–85 mm/s of variable speed
	Motor (geared motor):	Triple phase, 100 W, direction of rotation reversible under inverter control
Incision unit	CO_2 gas laser:	Maximum output 12 W, focal distance 190 mm, combined with a scanner, irradiation area 100 mm × 100 mm, wavelength 10.6 μm
	Laser controller:	Operation of the laser device with a console
	Sensor:	Proximity type for aluminum, detects work and transmits signals to the CO_2 laser to start laser irradiation
Collection unit	Collecting container:	Depth 15 mm × width 100 mm × length 4,090 mm Cold water at 5ºC is circulated in the bottom part
	Cooler:	Cold-water circulation type (37 L, range of temperature adjustment 10–25ºC) Cooling capacity 10.5×10^6 (J/h) at a liquid temperature of 20ºC The collecting container is cooled with 2 coolers
Removal unit	Silkworms are removed (released) from the holder by a cam follower and the cams of the holders and are recovered	
Size (mm)	3,700 (overall length) × 1,845 (overall width) × 1,350 (overall height)	

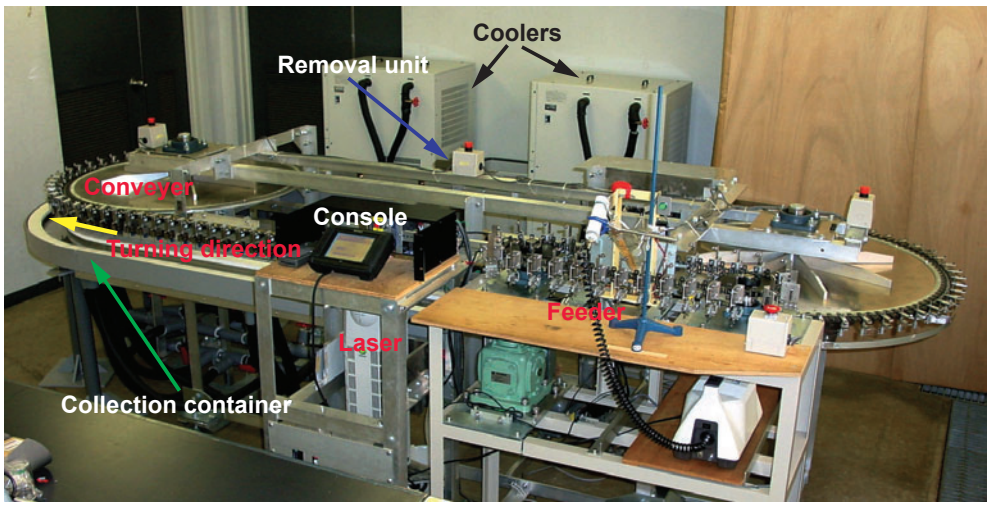

Fig. 1. Developed automatic silkworm larvae hemolymph collection system

was examined by using 4 and 5-day 5th-instar silkworm larvae (Ariake variety). Larvae were reared on artificial feed, and anesthetized at 0ºC for 20 min prior to use. Anesthetized larvae were continuously fed into the hemolymph collection system, and hemolymph was collected for 2 min after incision.

As for the operation conditions of hemolymph collection, the transport speed of the main conveyer, to which the modified silkworm holders were attached at intervals of 64 mm, was set at 32 mm/s, and the system was operated by one person. The laser output, scanning speed, and irradiation length were 6.5 W, 61.7 mm/s, and 14.5 mm, respectively.

Results and Discussion

The performance of each unit of the hemolymph collection system examined was as follows.

1. Feeding of silkworm larvae

By manually putting silkworms that had become half stiffened and rod-like under hypothermic anesthesia at 0ºC for 20 min[3] into the chute with the head first at a rate of one silkworm per 2 seconds, the silkworms took the supine position as they fell through the chute and reached the exit of the chute (Fig. 2). Then, the transfer arms attached to the sub-conveyer, which rotated toward the CO_2 laser, loaded by dropping the silkworms between the opened plate springs of the holders, which were horizontal on the main conveyer. The holders that received the silkworms held them by closing the springs, turned to the vertical position with the head of the silkworms up, and continuously carried them to the CO_2 laser (Fig. 3).

However, silkworms were occasionally blocked in

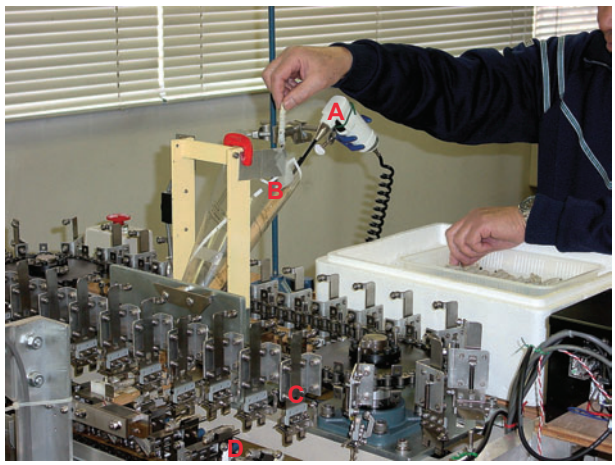

Fig. 2. Silkworm feeder unit
A: Air nozzle, B: Chute, C: Transfer arm, D: Holder.

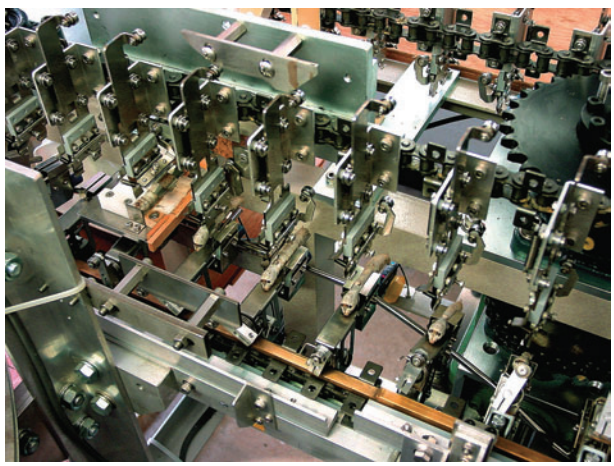

Fig. 3. Transfer of supplied silkworms after sliding down from chute

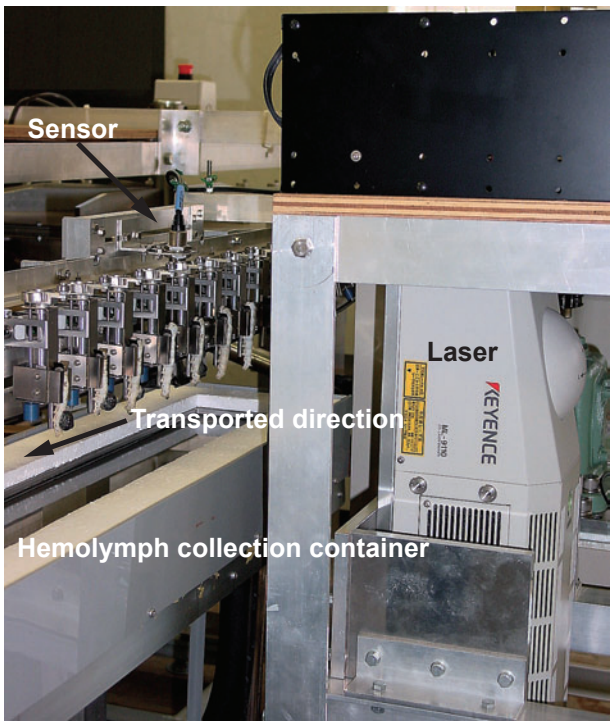

Fig. 4. Laser incision of silkworm larvae

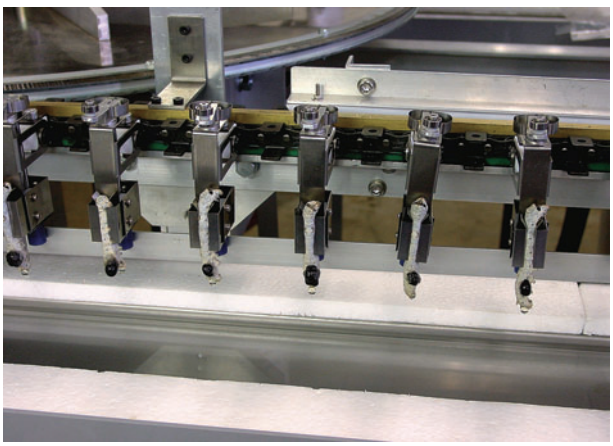

Fig. 5. Collection of hemolymph spouted from incised silkworms

the chute, because the body surface of the silkworms became wet by condensation and did not slide easily down the chute. The following measures were taken to solve this problem.

Low friction plastic tape (Commercial name: Shikii Suberi, 21 mm (W) × 350 mm (L), Kawaguchi Giken, Saitama, Japan) was attached to the slope surface of the chute for reducing the coefficient of friction. Also, the sliding speed of silkworms down the chute was increased by attaching an air nozzle to the entry of the chute which blows air into the chute at 0.1 MPa. This improved sliding of silkworms down the chute. Moreover, this kept the slope of the chute dry and resolved the problem of blocking. Particularly, since condensation on the body surface of silkworms is more likely to occur in wet seasons, dehumidification of the environment to humidity of 50%

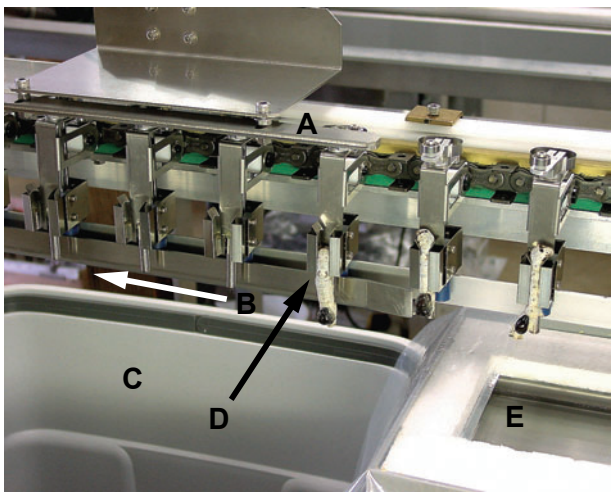

Fig. 6. Removal of silkworms from holders after collection of hemolymph
A: Cam follower for holder opening,
B: Direction of conveyer chain transportation,
C: Pail of abandoned silkworms,
D: Silkworm dropping from holder,
E: Terminal of hemolymph collection container.

Table 2. Amount of hemolymph collected by developed collection system vs. time after incision

Days in 5th instar	Sex	No. of larvae	Weight of silkworm (g)	Amount of hemolymph (Ratio to weight of silkworm) (%)				*Hemolymph collection ratio (A) (%)
				0–1 min	1–1.5 min	1.5–2 min	Total (T)	
5•4	♀	15	4.75	10.51	1.92	1.40	13.83	55.32
	♂	15	4.18	9.45	1.88	1.24	12.57	50.28
5•5	♀	15	5.22	8.80	2.02	1.67	12.49	50.96
	♂	15	4.57	8.26	1.93	1.65	11.84	47.36

Silkworm race: Ariake
*Hemolymph collection ratio was evaluated using next equation: A (%) = (T/25) × 100

or less with an air-conditioner is considered to be effective.

2. Laser incision and collection of hemolymph

As shown in Fig. 4, the holders that received the silkworms from the feeder moved to the front of the laser. Then, when the sensor mounted above the main conveyer detected a holder, the laser performed an incision of only the integument in the back of each silkworm. Next, the holders moved above the hemolymph collection container without dropping the silkworms, and hemolymph that spouted from the silkworms was collected as it dripped spontaneously into the container. After the incision, the state of the silkworms held with the holders is shown in Fig. 5. The integument of the 8th to the 10th segments was incised linearly over about 11–12 mm. However, because damage and dropouts occurred hardly, mixing of protein digestion enzymes of the midgut was avoided in the collection hemolymph, and it seems that high purity hemolymph collection could be achieved.

Table 2 shows the average amount of hemolymph from a silkworm (percentage of the body weight). In both males and females of 4-day 5th-instar silkworm, the amount of hemolymph was about 10% of the body weight 1 min after the incision, slightly less than 2% after 1–1.5 min, and slightly more than 1% after 1.5–2 min. The total amount came to about 13%. Similarly, in both males and females of 5-day 5th-instar silkworm, the amount was slightly more than 8% of the body weight during 1 min after the incision, about 2% after 1–1.5 min, and about 1.6% after 1.5–2 min. The total amount came to about 12%. At both days, more hemolymph could be collected from females than from males, and 70–80% of the total yield was obtained during the first minute of collection. Moreover, since the hemolymph content of silkworms at this age is reported to be about 25% of the body weight[1,5] the collection ratio estimated by dividing the total amount (T) by 25 was about 50% (= (T/25) × 100). On the other hand, the amount of hemolymph collection by the manual procedure is estimated to be 15–16% of the body weight (Kobayashi, unpublished). There seems to be room for improvement in the collection efficiency of this automatic mechanization system.

3. Removal of silkworms

After collection of hemolymph, the plate springs opened as the levers of the holders were pushed by the cam mechanism shown in Fig. 6. Then, the silkworms were released from the holders and they readily dropped into a pail by the force of self-weight. In this process, because the holders opened consistently, it is evaluated to be successful in detaching silkworms.

Summary and Conclusions

Using the newly developed hemolymph collection system, hemolymph could be collected continuously from 4 and 5-day 5th-instar silkworm larvae, and one-man operation of the system appeared to be possible. Moreover the total process of feeding silkworms into the system, laser incision, collection of hemolymph, and removal of larvae after the collection could be performed in less than 2.5 min per silkworm. In this study, silkworms that contracted virus after inoculation of recombinant BmNPV were not used, but in the insect factory, the hemolymph is collected at 4–5 days post-inoculation of 5th-instar, when the inoculated larvae begin to develop symptoms but are nearly as healthy as normal larvae. Therefore, this system is expected to function adequately when silkworms that have been infected with recombinant BmNPV are used. However, it is considered to be difficult to manually continue feeding 1 silkworm every 2 seconds to this system for a long time. So that, to put this system into practical use, improvement of the feeding efficiency is still needed by achieving further mechanization of supplying silkworms to the system. Although this system has been developed for silkworms, it can also be applicable to larvae and pupae of other insects.

In addition, the commercial market for interferon produced by an insect factory of a certain company is presently estimated to be 1 billion yen, and that for pet vaccines is about 2 billion yen. The market for medicines produced by insect factories is expected to enlarge to the order of at least 10 billion yen after 10 years. Therefore, the production of medicines that are needed in large quantities such as human influenza vaccines, allergic examination medicines and medicines for animals are expected to generate the future demand for this system using silkworms. Accordingly, the practical use of an immediate automatic hemolymph collecting system for silkworms will be strongly hoped for in the future.

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