J. Microbiol. Biotechnol. (2007), **17**(12), 1944–1948

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

© The Korean Society for Microbiology and Biotechnology

Effects of Silkworm Hemolymph on Cell Viability and hCTLA4Ig Production in Transgenic Rice Cell Suspension Cultures

CHEON, SU-HWAN, KYOUNG-HOON LEE, JUN-YOUNG KWON, HYUN-NAM RYU, DA-HYUN YU, YONG-SOO CHOI, AND DONG-IL KIM*

Department of Biological Engineering, Inha University, Incheon 402-751, Korea

Received: March 6, 2007 Accepted: July 10, 2007

Abstract Silkworm hemolymph (SH), prepared from fifth-instar larvae of *Bombyx mori* and heat-treated at 60°C for 30 min, was used to improve cell viability and the production of human cytotoxic T-lymphocyte antigen 4-immunoglobulin (hCTLA4Ig) in transgenic *Oryza sativa* L. cell suspension cultures. Even though SH could not elevate cell viability at the concentrations up to 3% (v/v), addition of 0.3% (v/v) SH to a culture medium enhanced the production of hCTLA4Ig by 36.8% over an SH-free medium. Moreover, the production period of hCTLA4Ig could be shortened in a 0.3% (v/v) SH-added medium compared with that in an SH-free culture. As a result, addition of 0.3% (v/v) SH improved the productivity of hCTLA4Ig significantly in transgenic rice cell cultures.

Keywords: Silkworm hemolymph (SH), transgenic plant cell cultures, hCTLA4Ig, RAmy3D promoter

Transgenic plant cell suspension cultures have many advantages over other host systems for industrial uses in the production of biopharmaceuticals. They have a low production cost and a capability for posttranslational modification including glycosylation [18]. In addition, they are safe to use in a human pathogen-free process and facilitate protein recovery [9]. Recently, rice cells have been genetically modified with the rice α-amylase 3D (RAmy3D) promoter to produce recombinant human proteins such as granulocyte-colony stimulating factor (G-CSF) [10], granulocyte-macrophage colony stimulating factor (GM-CSF) [24], serum albumin [11], α_1-antitrypsin [19, 27, 29], interleukin [15], and interferon-gamma [4]. In the RAmy3D promoter system, rice cells are commonly regulated by sugar starvation.

Human cytotoxic T-lymphocyte antigen 4-immunoglobulin (hCTLA4Ig) is a soluble fusion protein that combines the extracellular domain of the human CTLA-4 receptor and the Fc fragment of human immunoglobulin G, and is expected to be a potent antigen-specific immunosuppressive drug [1]. hCTLA4Ig binds to CD80 and CD86 on antigen-presenting cells and delivers an inhibitory signal that downregulates T cell activation [16]. Orencia (abatacept), a recombinant hCTLA4Ig produced by animal cell cultures, was approved by the FDA in December 2005 for the treatment of rheumatoid arthritis [3]. Recently, hCTLA4Ig was expressed in transgenic rice cell suspension cultures and its characteristics and *in vivo* activities were investigated [17]. Because hCTLA4Ig is inducibly produced under the control of the RAmy3D promoter by sugar starvation, the production phase could be separated from the cell growth phase to effectively enhance the productivity of hCTLA4Ig. This enhancement, which can be achieved by a medium exchange method that depletes sugar in a medium, may induce cell death because of the loss of carbon sources for the energy metabolism. It may also cause cell rupture as a result of changes in the osmotic pressure. Hence, various unfavorable factors may stimulate apoptosis, and proteases may accumulate in cells and be secreted into the culture medium, thereby reducing the hCTLA4Ig production during the production phases.

Silkworm hemolymph (SH), prepared from *Bombyx mori*, is an insect serum that has been widely used as a medium supplement in insect or animal cell cultures because it can be prepared easily and economically [5, 8, 12]. In addition, it contains many biologically active components such as proteins, carbohydrates, lipids, and small molecules [23]. Those are antiapoptotic 30 K proteins [13], proteinaceous protease inhibitor [25], cysteine proteinase [32], glucose-binding 30 K lipoproteins [30], as well as trehalose and glucose for storage carbohydrates [22]. Moreover, hemolymph of silkworm reportedly increases the production of a recombinant protein and enhances cell growth [7, 31].

Until now, there have been no researches on SH to increase the cell viability and productivity of a recombinant protein in plant cell suspension cultures. In this study, we have therefore evaluated the feasibility of applying SH to

*Corresponding author
Phone: 82-32-860-7515; Fax: 82-32-875-0827;
E-mail: kimdi@inha.ac.kr

enhance the cell viability and the production of hCTLA4Ig in transgenic rice cell suspension cultures.

MATERIALS AND METHODS

Cell Cultures

Transgenic rice suspension cells (*Oryza sativa* L. cv. Dongjin) producing hCTLA4Ig under the control of the RAmy3D promoter were obtained by a particle bombardment method [16]. Established cells were subcultured every 10 days. Subcultures were performed with 20% (v/v) inoculum, and the cells were cultured on a shaker at 110 rpm and incubated at 27°C in the dark using AA medium [28] containing 30 g/l sucrose, 2 mg/l 2,4-dichlorophenoxyacetic acid, and 0.02 mg/l kinetin.

Preparation of SH

SH was collected from the fifth-instar larvae of *Bombyx mori* by clipping the side of an abdominal leg [5]. On average, 0.5 ml of hemolymph was obtained per larva. The collected hemolymph was heat-treated at 60°C for 30 min, and then chilled and centrifuged at 12,000 rpm for 30 min [8]. Finally, the supernatant was filtered through a 0.2-μm membrane filter and added directly to the medium.

Estimation of Cell Growth

The cell suspension was filtered through a Whatman No. 1 filter paper, under vacuum, and washed three times with distilled water to remove residual sugars from the cells surfaces. The cells were then transferred to preweighed dishes, and the total mass measured. The dry cell weight (DCW) was estimated after drying for 2 days at 60°C.

Measurement of Cell Viability

The cell viability was evaluated by the TTC (2,3,5-triphenyl-tetrazolium chloride) method [26]. One percent (w/v) TTC in a 0.05 M KH_2PO_4 buffer (pH 9.0) was added to 0.5 g of wet cells. The cells were then incubated at 20°C under dark conditions for 24 h. The samples were centrifuged at 12,000 rpm for 15 min and the resulting supernatant was collected. After applying 3 ml of 95% (v/v) ethanol to extract the red formazan in the viable cells, the samples were placed in a 60°C water bath for 30 min. After cooling at room temperature, the samples were centrifuged again at 15,000 rpm for 30 min to obtain the supernatant. The absorbance of the extracted formazan was measured at 485 nm using an Agilent 8453 spectrophotometer (Agilent Technologies Inc., U.S.A.) and the relative cell viability was estimated.

Determination of the Recombinant hCTLA4Ig

The hCTLA4Ig expression level was measured by enzyme-linked immunosorbent assay (ELISA). Suspension-cultured medium was centrifuged at 4°C and at 12,000 rpm. The supernatant was stored at −70°C until use. For sandwich ELISA, 96-well microplates were coated with goat anti-human IgG Fc (1:1,000; KPL Inc., U.S.A.) and blocked with phosphate-buffered saline containing 2% fetal bovine serum and 0.05% Tween 20. Each well was loaded with sample or protein standard ranging from 11 to 0.08 ng/ml, serial of eight, each at two-fold dilution. The application of peroxidase-labeled goat anti-human IgG (g) (1:5,000; KPL Inc., U.S.A.), used as the detection antibody, was followed by horseradish-peroxidase (HRP) substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; KPL Inc., U.S.A.). A Model 550 microplate reader (Bio-Rad Laboratories Inc., U.S.A.) was used to measure the absorbance of the developed color at 405 nm.

Protease Assay

The extracellular protease activity was measured by a modification to Anson's method [2]. The reaction solution (0.5 ml) contained 1% (w/v) of Na-caseinate as the substrate, in 67 mM phosphate buffer (pH 7.0), with the addition of 0.5 ml to the prewarmed medium sample. Tyrosine at 0.4 mg/l was used as a standard. The reaction solution and sample mixture was incubated at 50°C for 10 min, and the reaction terminated by the addition of 0.3 ml of 30% trichloroacetic acid (TCA). The mixture was allowed to stand for 15 min, and then centrifuged at 12,000 rpm for 10 min. The absorbance of the developed color was measured at 280 nm, which provided the tyrosine concentration, using an Agilent 8453 spectrophotometer (Agilent Technologies Inc., U.S.A.). One unit of protease activity was defined as the amount of enzyme that liberated 1 μg of tyrosine per min, under the specified conditions.

Measurement of Total Protein

The total soluble protein in the medium was estimated using the Bradford method, with bovine serum albumin as a standard. The absorbance at 595 nm was detected using a Model 550 microplate reader (Bio-Rad Laboratories Inc., U.S.A.).

RESULTS

Effects of SH on Protease Activity, hCTLA4Ig Production, and Relative Viability

The protease activity was estimated at 40.2 U/ml in 3% SH-added medium, and 0 U/ml in SH-free medium at day 0 as shown in Table 1. The total protein level was 132.7 mg/l in 3% SH-added medium, and 0 mg/l in SH-free medium at day 0. The pH value rose from 5.8 in 3% SH-added medium to 6.3 in SH-free medium. Various concentrations of SH, ranging from 0.03% to 3% (v/v), were used to investigate the effects of SH on cell growth, the production of hCTLA4Ig, and viability (Fig. 1). The cell mass of the

Table 1. Comparisons of the properties of SH-added medium and SH-free medium for pH, total protein, and protease activity.

Types	pH	Total protein (mg/l)	Protease activity (U/ml)
SH-free medium	5.8	0	0
SH-added medium (3%)	6.3	132.7	40.2

control culture in SH-free medium decreased sharply from 7.49 gDCW/l at day 0 to 4.67 gDCW/l at day 4 because of the depletion of sucrose in the medium during the induction phase. The cell mass in 0.3% SH-added culture decreased slowly to 5.40 gDCW/l at day 4. In terms of the production of recombinant protein, the hCTLA4Ig level with 0.3% SH (33.8 mg/l) was 36.8% higher than that of the control (24.7 mg/l) at day 4. Relative viability with 0.3% SH increased to 5.47% compared with that of the control at day 8.

Time-Course Changes of hCTLA4Ig Production

To evaluate the effect of SH on hCTLA4Ig production according to the progress of production phase, time-course behaviors were observed after induction. As shown in Fig. 2, the production of hCTLA4Ig increased steadily during the induction periods. In SH-free medium, the production of hCTLA4Ig was 13.8 mg/l at day 2, and 25.7 mg/l at day 4. Addition of 0.3% SH significantly improved the production of hCTLA4Ig. The maximum level of hCTLA4Ig was 33.79 mg/l in the 0.3% SH-added medium, and 25.73 mg/l of SH-free medium at day 4. However, addition of 0.03 or 3% SH into the medium could not increase the production of hCTLAIg remarkably in suspension cultures of transgenic rice cells. Moreover, the induction period of hCTLA4Ig was about 2 days shorter for the 0.3% SH-added medium than for the SH-free medium. As shown in Fig. 2, it was found that the use of 0.3% SH provided the highest level of hCTLA4Ig at day 4.

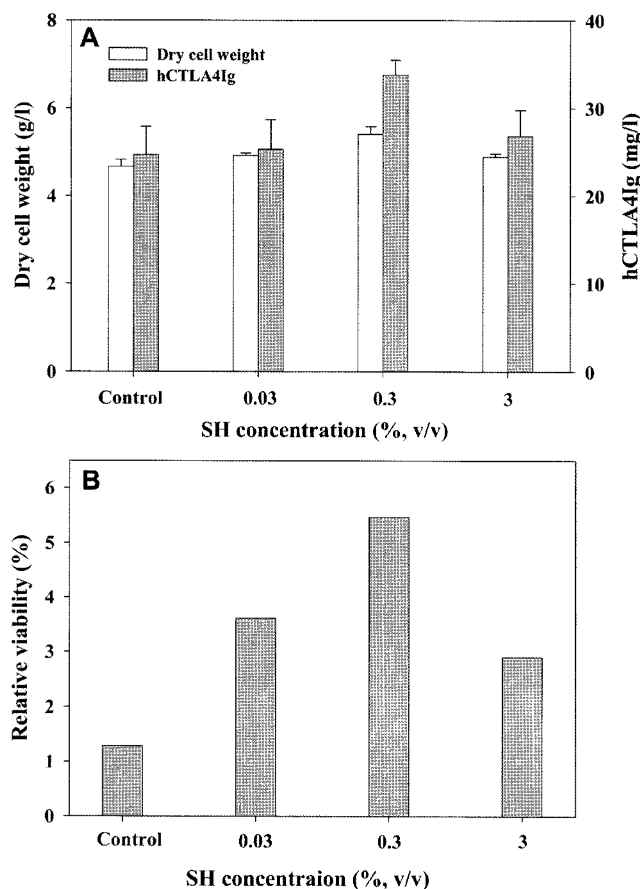

Fig. 1. Effects of the different concentrations of SH on (**A**) cell mass, hCTLA4Ig production, and (**B**) relative viability.
Sampling was performed at the time of induction (day 0), 4 days for cell mass and hCTLA4Ig production, and 8 days for relative viability.

DISCUSSION

Recently, SH has been used as a medium supplement in animal cell cultures for three purposes: for the prevention of apoptosis [7], for the improved production of recombinant proteins [8], and for the stimulation of cell growth. Until now, there have been no studies on the use of SH for the production of a recombinant protein in transgenic plant cell cultures. Since SH has high protease activity, we contemplated applying SH to plant cell cultures so as not to denature and degrade the recombinant protein. Nonetheless, it was reported that the addition of SH to a growth medium on a CHO cell system inhibited sodium butyrate-induced apoptosis and produced a five-fold increase in the volumetric productivity of erythropoietin [7]. Upon addition of SH into transgenic rice cell cultures, positive effects on the production of hCTLA4Ig and the longevity of induced plant cells could be noticed. The expression of 30 K proteins derived

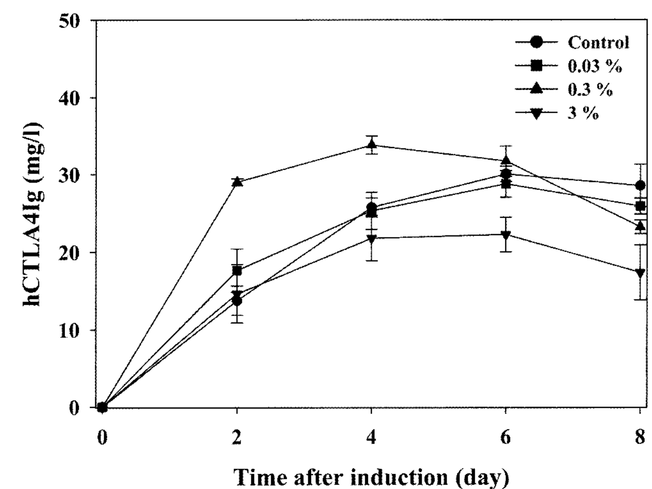

Fig. 2. Time profiles for the production of hCTLA4Ig during the production phase with various concentrations of SH.

from SH effectively increased the longevity of the host cell [13]. The 30 K proteins, called storage proteins, are a group of structurally related proteins with a molecular weight of approximately 30 K and they comprise 11% of all the proteins in SH [14].

Unlike our expectations, SH failed to inhibit cell death in transgenic rice cell suspension cultures after induction. Studies have shown that the addition of SH to a culture medium successfully delays apoptosis in a CHO cell system [7, 21] and in an insect cell-baculovirus system [8]. Furthermore, the addition of purified 30 K protein to a culture medium effectively prolongs the cell viability of the insect cell Sf9 [20] and the mammalian cell HeLa [6]. In contrast to these studies, the SH-added medium that we used in all samples from 0.03% to 3% showed no improvement of cell viability in transgenic plant cell suspension cultures after induction. Differences in the antiapoptotic effect of the 30 K protein between plant cells and a mammalian cell system (or an insect cell system) can be postulated. We also inferred that the treatment conditions for inducing apoptosis in a mammalian cell system would be different from those in a transgenic rice cell system. The former induces apoptosis through the sodium butyrate, camptothecin, staurosporine, and actinomycin that exist in the carbon sources of energy metabolism. The latter causes cell death by depleting the sugar in the medium without supplying energy sources. Thus, to improve cell viability in transgenic plant cell suspension cultures, we need to remove a considerable portion of the proteases in the SH and to purify the 30 K proteins.

How does SH enhance the production of hCTAL4Ig with the RAmy3D promoter in transgenic rice cell cultures? The RAmy3D promoter is strongly regulated by sugar starvation. The expression of hCTLA4Ig with the aid of the RAmy3D promoter is influenced differently by the types and concentrations of sugar [27]. Furthermore, the SH prepared from fifth-instar larvae of *Bombyx mori* contains trehalose and glucose for storage [22] as well as glucose-binding proteins (30 K lipoproteins) [30]. However, when small amounts of sugar, such as sucrose, glucose, or trehalose, are included in the SH-added medium as an energy source, the relative cell viability declines steadily, but not so rapidly. Hence, the SH-added medium is unlikely to contain sugar in all samples. It is likely that the 30 K lipoproteins in the SH-added medium could bind to glucose in cells and stimulate the synthesis of hCTLA4Ig by using the RAmy3D promoter, which is repressed by glucose. In conclusion, SH could be a useful additive for enhancing the production of recombinant proteins in a transgenic rice cell culture system using the RAmy3D promoter.

Acknowledgment

This work was supported by an Inha Research Grant.

REFERENCES

1. Abrams, J. R., M. G. Lebwohl, C. A. Guzzo, B. V. Jegasothy, M. T. Goldfarb, B. S. Goffe, A. Menter, N. J. Lowe, G. Krueger, M. J. Brown, R. S. Weiner, M. J. Birkhofer, G. L. Warner, K. K. Berry, P. S. Linsley, J. G. Krueger, H. D. Ochs, S. L. Kelley, and S. Kang. 1999. CTLA4Ig-mediated blockade of T-cell costimulation in patients with *Psoriasis vulgaris*. *J. Clin. Invest.* **103:** 1243–1252.
2. Battaglino, R. A., M. Heurgo, A. M. R. Pilosof, and G. B. Bartholomai. 1991. Culture requirements for the production of protease by *Aspergillus oryzae* in solid state fermentation. *Appl. Microbiol. Biotechnol.* **35:** 292–296.
3. Bluestone, J. A., E. W. St. Clair, and L. A. Turka. 2006. CTLA4Ig: Bridging the basic immunology with clinical application. *Immunity* **24:** 233–238
4. Chen, T. L., Y. L. Lin, Y. L. Lee, N. S. Yang, and M. T. Chan. 2004. Expression of bioactive human interferon-gamma in transgenic rice cell suspension cultures. *Transgenic Res.* **13:** 499–510.
5. Chen, Y., Z. Zhu, X. Lin, Y. Yi, Z. Zhang, and G. Shen. 2005. Overexpression and characterization of appA phytase expressed by recombinant baculovirus-infected silkworm. *J. Microbiol. Biotechnol.* **15:** 466–471.
6. Choi, S. S., W. J. Rhee, and T. H. Park. 2002. Inhibition of human cell apoptosis by silkworm hemolymph. *Biotechnol. Prog.* **18:** 874–878.
7. Choi, S. S., W. J. Rhee, and T. H. Park. 2005. Beneficial effect of silkworm hemolymph on a CHO cell system: Inhibition of apoptosis and increase of EPO production. *Biotechnol. Bioeng.* **91:** 793–800.
8. Ha, S. H. and T. H. Park. 1997. Efficient production of recombinant protein in *Spodoptera frugiperda*/AcNPV system utilizing silkworm hemolymph. *Biotechnol. Lett.* **19:** 1087–1091.
9. Hellwig, S., J. Drossardk, R. M. Twyman, and R. Fisher. 2004. Plant cell cultures for the production of recombinant proteins. *Nature Biotechnol.* **22:** 1415–1422.
10. Hong, S. Y., T. H. Kwon, Y. S. Jang, S. H. Kim, and M. S. Yang. 2006. Production of bioactive human granulocyte-colony stimulating factor in transgenic rice cell suspension cultures. *Protein Expr. Purif.* **47:** 68–73.
11. Huang, L. F., Y. K. Liu, C. A. Lu, S. L. Hsieh, and S. M. Yu. 2005. Production of human serum albumin by sugar starvation induced promoter and rice cell culture. *Transgenic Res.* **14:** 569–581.
12. Kim, E. J. and T. H. Park. 1999. Reduction of FBS concentration through adaptation process in mammalian cell culture and addition of silkworm hemolymph in insect cell culture. *J. Microbiol. Biotechnol.* **9:** 227–229.
13. Kim, E. J. and T. H. Park. 2004. Increase of host cell longevity by the expression of 30 K protein originating from silkworm hemolymph in an insect cell-baculovirus system. *Enzyme Microb. Technol.* **35:** 581–586.
14. Kim, E. J., W. J. Rhee, and T. H. Park. 2001. Isolation and characterization of an apoptosis-inhibiting component from the hemolymph of *Bombyx mori*. *Biochem. Biophys. Res. Commun.* **285:** 224–228.

15. Lee, B. Y., J. H. Lee, H. S. Yoon, K. H. Kang, K. N. Kim, J. H. Kim, J. K. Kim, and J. K. Kim. 2005. Expression of human interleukin-11 and granulocyte-macrophage colony-stimulating factor in transgenic plants. *J. Microbiol. Biotechnol.* **15:** 1304–1309.
16. Lee, K. H., H. J. Hong, S. M. Lim, S. H. Cheon, and D. I. Kim. 2006. Expression of human T-lymphocyte antigen 4 (hCTLA4) fused with hepatitis B surface antigen (HBsAg) using *Pichia pastoris*. *Enzyme Microb. Technol.* **39:** 481–486.
17. Lee, S. J, C. I. Park, M. Y. Park, H. S. Jung, W. S. Ryu, S. M. Lim, H. K. Tan, T. H. Kwon, M. S. Yang, and D. I. Kim. 2007. Production and characterization of human CTLA4Ig expressed in transgenic rice cell suspension cultures. *Protein Expr. Purif.* **51:** 293–302.
18. Lee, S. Y. and D. I. Kim. 2006. Perfusion cultivation of transgenic *Nicotiana tabacum* suspensions in bioreactor for recombinant protein production. *J. Microbiol. Biotechnol.* **16:** 673–677.
19. McDonald, K. A., L. M. Hong, D. M. Trombly, Q. Xie, and A. P. Jackman. 2005. Production of human α_1-antitrypsin from transgenic rice cell culture in a membrane bioreactor. *Biotechnol. Prog.* **21:** 728–734.
20. Rhee, W. J., E. J. Kim, and T. H. Park. 2002. Silkworm hemolymph as a potent inhibitor of apoptosis in Sf9 cells. *Biochem. Biophys. Res. Commun.* **295:** 779–783.
21. Rhee, W. J. and T. H. Park. 2001. Flow cytometric analysis of the effect of silkworm hemolymph on the baculovirus-induced insect cell apoptosis. *J. Microbiol. Biotechnol.* **11:** 853–857.
22. Satake, S., Y. Kawabe, and A. Mizoguchi. 2000. Carbohydrate metabolism during starvation in the silkworm *Bombyx mori*. *Arch. Insect Biochem.* **44:** 90–98.
23. Seong, S. I. 1986. Biochemical studies on the major hemolymph proteins (MHPs) of the silkworm, *Bombyx mori*. *Kor. J. Seric. Sci.* **28:** 30–36.
24. Shin, Y. J., S. Y. Hong, T. H. Kwon, Y. S. Jang, and M. S. Yang. 2003. High level of expression of recombinant human granulocyte-macrophage colony stimulating factor in transgenic rice cell suspension culture. *Biotechnol. Bioeng.* **82:** 778–783.
25. Shirai, K., H. Fujii, H. Doira, and H. Iwamoto. 2000. Synthesis and resorption of a humoral chymotrypsin inhibitor, CI-8, by fat body of the silkworm, *Bombyx mori*. *Insect Biochem. Molec.* **30:** 363–368.
26. Steponkus, P. L. and F. O. Lanphear. 1967. Refinement of the triphenyl tetrazolium chloride method of determining cold injury. *Plant Physiol.* **42:** 1423–1426.
27. Terashima, M., Y. Ejiri, N. Hashikawa, and H. Yoshida. 2000. Effects of sugar concentration on recombinant human α_1-antitrypsin production by genetically engineered rice cell. *Biochem. Eng. J.* **6:** 201–205.
28. Thompson, J. A., R. Abdullah, and E. C. Cocking. 1986. Protoplast culture of rice (*Oryza sativa* L.) using media solidified with agarose. *Plant Sci.* **47:** 123–133.
29. Trexler, M. M., K. A. McDonald, and A. P. Jackman. 2002. Bioreactor production of human α_1-antitrypsin using metabolically regulated plant cell cultures. *Biotechnol. Prog.* **18:** 501–508.
30. Ujita, M., A. Kimura, D. Nishino, E. Yokoyama, Y. Banno, H. Fujii, and A. Hara. 2002. Specific binding of silkworm *Bombyx mori* 30-kDa lipoproteins to carbohydrates containing glucose. *Biosci. Biotechnol. Biochem.* **66:** 2264–2266.
31. Woo, S. D., H. S. Kim, W. J. Kim, B. R. Jin, and S. K. Kang. 1995. Enhanced expression of foreign gene in baculovirus-infected insect cells using a silkworm hemolymph. *Kor. J. Seric. Sci.* **37:** 181–185.
32. Yamamoto, Y., S. Watabe, T. Kageyama, and S. Y. Takahashi. 1999. Purification and characterization of *Bombyx* cysteine proteinase specific inhibitors from the hemolymph of *Bombyx mori*. *Arch. Insect Biochem.* **41:** 119–129.