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Oxidation-Deficient Silkworm Hemolymph as a Medium Supplement for Insect Cell Culture

Eun Jeong Kim[1], Ji-Young Choi[2], Sam-Eun Kim[2], and Tai Hyun Park[1]*

[1]School of Chemical Engineering, Seoul National University, Seoul 151-742, Korea
[2]Department of Sericulture and Entomology, National Institute of Agricultural Science and Technology, Suwon 441-100, Korea

Hemolymph is oxidized and darkens visibly during the collection from silkworms due to the activity of tyrosinase in it. Toxic quinones are produced by the oxidation and consequently inhibit the cell growth. Heat treatment can be used to prevent the oxidation; however, the oxidation may occur during the collection of hemolymph before it is heat-treated. It makes the hemolymph collection difficult especially on a large-scale preparation. Hemolymphs collected from 257 different strains of silkworms were examined to select the slowly oxidized hemolymphs. Hemolymphs collected from mutant strains such as Lemone, TBO, Cre, Y4, and wE^b showed relatively slow color changes. Oxidation rates of the hemolymphs were measured by the absorbance change using a spectrophotometer. The hemolymph of wE^b showed the slowest oxidation. The absorbance of this mutant hemolymph reached the saturation value at 20℃ in 450 min, whereas the total oxidation time of the wild-type (Baekokjam) hemolymph at the same temperature was 120 min. We tested if this mutant hemolymph is useful as a medium supplement for insect cell culture. Cell growth rate and final cell concentration in the medium supplemented with the wE^b hemolymph were almost same as those in the medium supplemented with the wild-type hemolymph. Hemolymph is collected on a small scale by clipping the abdominal leg; however, this method is not appropriate for large scale preparation. Centrifugation after chopping the silkworm hemolymph by a blending mixer is a more appropriate procedure for large scale collection. Slowly oxidized wE^b hemolymph resulted in higher cell concentration than the wild-type hemolymph when hemolymph was collected by the large scale preparation method.

Key words: silkworm, hemolymph, insect cell, oxidation, medium supplement

INTRODUCTION

The insect cell-baculovirus system has advantages for the production of recombinant proteins since it has a strong polyhedrin promoter and proper post-translational modifications. One of the key issues of the insect cell-baculovirus system is the development of low serum or serum-free media [1]. In the early stages of insect tissue culture development, silkworm hemolymph was used as a culture medium. Based on the chemical analysis of the hemolymph [2], Wyatt [3] formulated a synthetic medium. It was improved by Grace [4]; however, Grace's medium still had to be supplemented with silkworm hemolymph. Even since fetal bovine serum (FBS) was proven to be beneficial for the growth of the insect cells, insect cell medium has been supplemented with FBS instead of insect hemolymph.

Although FBS contains a large number of different growth-promoting activities in a physiologically balanced blend, there are some drawbacks including high cost, lot-to-lot variation, undefined composition, increased contamination risk from mycoplasma, and the complication of downstream processing due to a high protein concentration [5]. In spite of many attempts to develop serum-free medium, the medium is supplemented with 10% FBS in most cases. FBS is a costly component, accounting for about 90% of the cost when the medium is supplemented with 10% FBS, whereas silkworm hemolymph is very cheap in Asia and can be easily collected since silkworm is a domesticated insect. The hemolymph is also not subject to nonreproducibility due to lot-to-lot variation since the silkworm is genetically stable and characterized. Recently it was shown that FBS concentration in the medium could be reduced to 1% without decrease in cell growth rate and maximum cell concentration by adding 5% silkworm hemolymph [6]. Moreover, the addition of silkworm hemolymph increased the production of recombinant protein [7].

Silkworm hemolymph is oxidized and darkens visibly and heat treatment is necessary to prevent

*Corresponding author
Tel: 02-880-8020 Fax: 02-888-7295
e-mail: thpark@plaza.snu.ac.kr

the oxidation since the oxidation results in the inhibition of cell growth. The oxidation may occur during the collection of silkworm hemolymph even before it is heat-treated. It makes the large-scale process for collecting hemolymph difficult. Thus, this work concerned with the development of oxidation-deficient silkworm hemolymph as a medium supplement for insect cell culture.

MATERIALS AND METHODS

Cell Line and Culture Condition

Spodoptera frugiperda (*Sf* 9) cells were grown in Grace's medium (Gibco) supplemented with 0.35 g/L $NaHCO_3$ and 1% antibiotic-antimycotic (Gibco). FBS and silkworm hemolymph were also added to the medium. Cells were grown at pH 6.2 and 28℃ in 25-cm^2 tissue culture flasks (Falcon) containing 6 mL medium.

Hemolymph Collection and Assay

Hemolymph was collected from 5th instar larvae by two different methods. The abdominal leg clipping method [6] was used for the small scale collection of hemolymph. For the large scale collection of hemolymph, silkworms were frozen at -20℃, then chopped by a blending mixer, and centrifuged at 12,000 rpm for 30 min. The hemolymphs collected by both methods were heat-treated at 60℃ for 30 min, then chilled and centrifuged. The supernatant was filtered with 0.2 μ membrane filter and used for supplementing the medium. The oxidation of hemolymph was analyzed by measuring the absorbance change at 400 nm using a spectrophotometer. The cell concentration was measured using a hemocytometer and cell viability was determined by trypan blue exclusion test [8]. Since dead cells absorb trypan blue (Sigma), they could be identified under light microscopy. Ten or more culture flasks were prepared initially for one set of experiments. After taking a sample every day, the flask was discarded and the next one used for the next sample.

RESULTS AND DISCUSSION

During the collection of silkworm hemolymph, it darkens visibly due to the activity of tyrosinase in hemolymph, producing melanin via intermediary quinones [3]. Melanin is a phenolic biopolymer widely distributed in nature. Eumelanins are brown and black while pheomelanins are yellow and red-brown. The color change of the hemolymph to dark brown implies that the melanin synthesized in the hemolymph is an eumelanin. Eumelanin is synthesized from tyrosine and its hydroxylated derivative dopa through a series of reactions [9, 10] as shown in Fig. 1. Melanin is biosynthesized from tyrosine through (i) hydroxylation of tyrosine to dopa, (ii) oxidation of dopa to dopaquinone, (iii) cyclization of dopaquinone to leucochrome, (iv) oxidative generation of dopachrome, (v) conversion of dopachrome to 5,6-dihydroxyindole, and (vi) oxidative polymerization of dihydroxyindole. Toxic quinones are produced in the series of reactions, and consequently inhibit the cell growth. The first two steps are catalyzed by tyrosinase while the rest of the steps are believed to occur nonenzymatically [11]. Therefore, several tyrosinase inhibitors and heat treatment have been suggested for the inactivation of tyrosinase. Wyatt reported that heat treatment could be successfully used at 60℃ for 5 min [3]; however, 5 min was not enough to prevent darkening of silkworm hemolymph in our experiment [6]. Thirty minute heat treatment at 60℃ rather than 5 min gave satisfactory results. Cells did not grow in the medium supplemented with non-treated hemolymph, whereas they grew with the hemolymph heat-treated for 30 min or longer [6]. Although the heat treatment is useful for the prevention of oxidation, silkworm hemolymph which does not occur the oxidation reaction, is more desirable. Because the oxidation is occurring quickly during the collection of silkworm hemolymph even before it will be heat-treated, especially on a large scale collection.

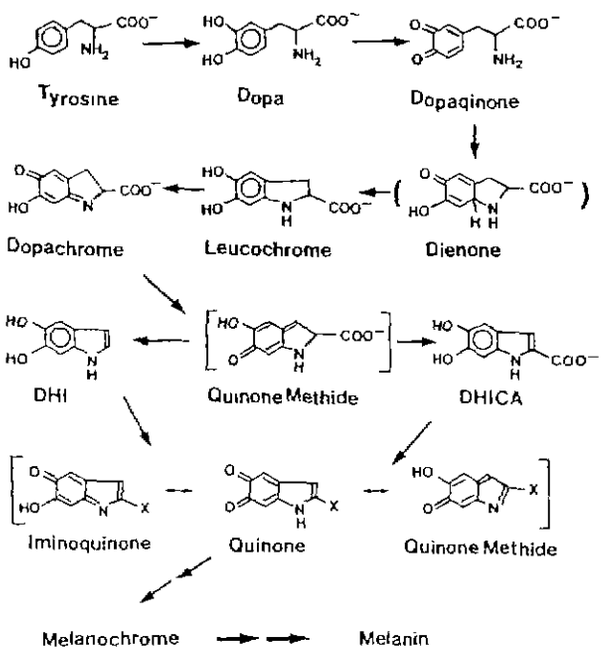

Fig. 1. Raper-Mason pathway for melanin biosynthesis (DHI=5,6-dihydroxyindole; DHICA=5,6-dihydroxyindole-2-carboxylic acid).

We have collected a variety hemolymphs from 275 different strains of silkworms reserved in National Institute of Agricultural Science and Technology, and examined the color change when exposed to the air. The color change represents the formation of an eumelanin through the oxidation reactions. Thus, we used the absorbance change as the extent of oxidation. Among them, five strains of which color of hemolymphs changed slowly, were selected. Those were Lemone, TBO, Cre, Y4, and wE^b. Oxidation rates of hemolymphs collected from these mutants were measured using a spectrophotometer. Fig. 2 and 3 show the absorbance changes of wild-type silkworm (Baekokjam) hemolymph at various wave lengths in a visible range and uv range, respectively. The absorbance in a visible range increases monotonically while the absorbance fluctuation was observed in a uv range. The fluctuation in a uv range

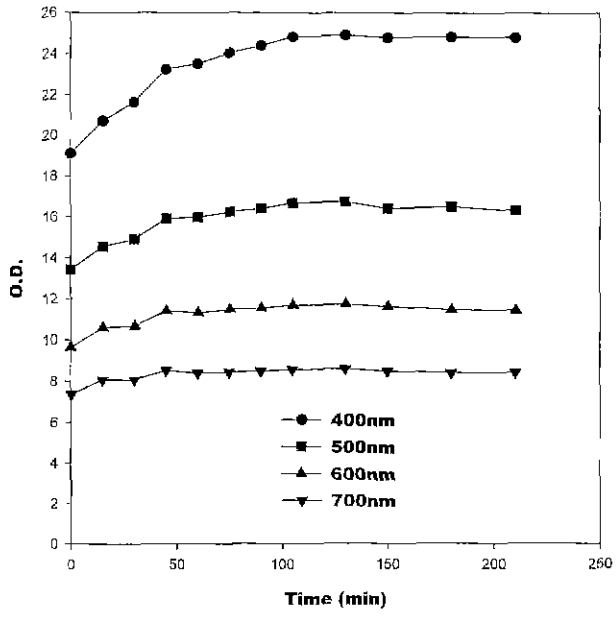

Fig. 2. Absorbance change of silkworm hemolymph in a visible range (Baekokjam).

occured in the mutant hemolymph. This fluctuation is caused by the formation and conversion of various intermediates shown in Fig. 1. Therefore, visible range is rather appropriate to measure the oxidation rate than uv range. As shown in Fig. 2, the absorbance chages in a visible range reach the saturation level in 120 min and 400 nm can be used as the most sensitive wave length for measuring the extent of oxidation.

The absorbance changes of mutant strains hemolymphs at 400 nm are shown in Fig. 4. Each absorbance was normalized by each final saturation value since the initial absorbance itself was different each other. The hemolymph of wE^b shows the slowest oxidation. The absorbance of this mutant hemolymph reaches the saturation level at 20℃ in 450 min, whereas the complete oxidation time of wild-type hemolymph at the same temperature is 120 min. From the data of Fig. 4, the complete oxidation time of each hemolymph is summarized in Table 1. Every hemolymph from mutant silkworms tested in this experiment shows total oxidation time at least twice as long as that of wild-type hemolymph. In the case of wE^b, the oxidation time is 3.75-fold longer. Fig. 4 represents that wE^b hemolymph has linear profile indicating zero order oxidation kinetics while the oxidation of wild-type hemolymph is higher order. Among the mutant silkworms used, wE^b showed the slowest oxidation of hemolymph. Insect cell growth was compared in Grace's medium supplemented with the wild-type hemolymph or wE^b hemolymph which were collected by clipping an abdominal leg. Fig. 5 shows that the wE^b hemolymph as good as the wild-type hemolymph as a medium supplement. Cell growth rate and final cell concentration are almost same in two cases. This means that the wE^b hemolymph is not deficient in the components for insect cell growth.

Hemolymph was collected on a small scale by clipping an abdominal leg; however, this method is not appropriate for large scale collection. Centrifugation after chopping the silkworm by a blending mixer and then heat treatment is a more appropriate procedure for large scale collection. Since the

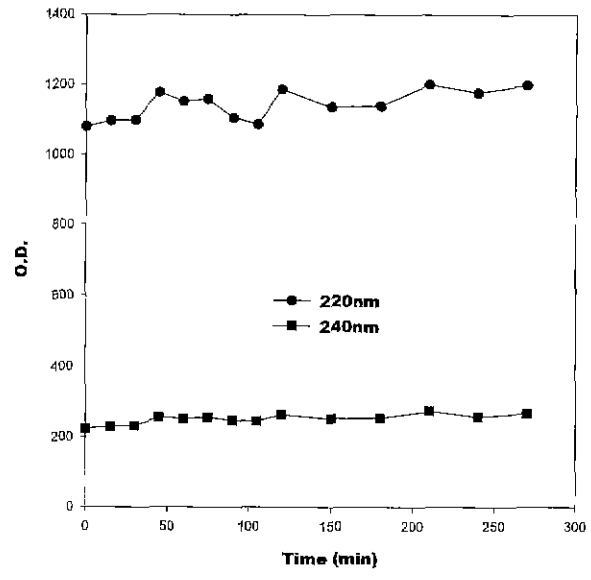

Fig. 3. Absorbance change of silkworm hemolymph in a UV range (Baekokjam).

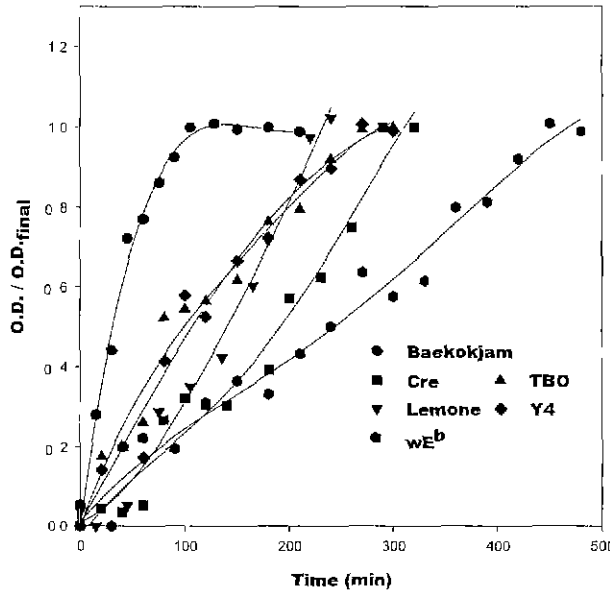

Fig. 4. Oxidation rate of hemolymph.

Table 1. Complete oxidation times of various hemolymphs.

Silkworm Types	Complete Oxidation Time (min)
Baekokjam	120
Lemone	240
Y4	270
Cre	290
TBO	300
wE^b	450

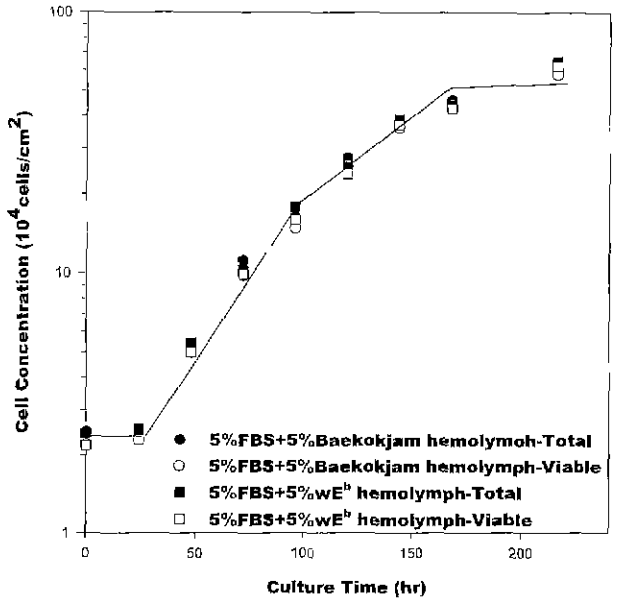

Fig. 5. Comparison of *Sf* 9 cell growth in the medium containing 5% Baekokjam hemolymph and 5% wE^b hemolymph.

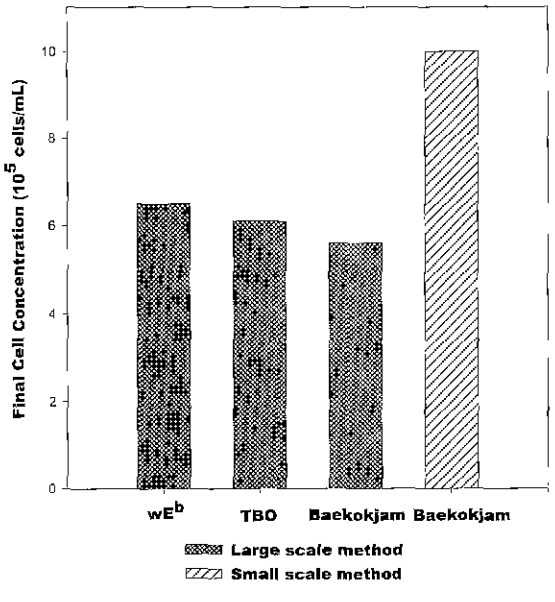

Fig 6. Insect cell growth in various hemolymphs collected by large scale method.

toxic oxidation occurs quickly during the collection of hemolymph before heat treatment, slowly oxidized hemolymph is more useful in this process. Fig. 6 shows the final cell concentrations grown in 24 well plates containing media supplemented with various hemolymphs which were collected by this process. The wE^b and TBO hemolymphs resulted in higher cell concentration than the wild-type hemolymph (Baekokjam). Therefore, oxidation-deficient hemolymphs are more useful than the wild-type hemolymph for the practical use. However, they still showed lower cell concentrations than the wild-type hemolymph collected by the small scale preparation method. Research for elucidating this reason is in progress.

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