Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–5, 2017

www.elsevier.com/locate/jbiosc

Chitin synthesis by *Chlorella* cells infected by chloroviruses: Enhancement by adopting a slow-growing virus and treatment with aphidicolin

Numfon Rakkhumkaew,[1] Takeru Kawasaki,[2] Makoto Fujie,[2] and Takashi Yamada[2,*]

Center for Research and Development of Agricultural Industry, Faculty of Agro Industry Product and Innovation Technology, Srinakarinwirot University, Ongkarak 26120, Thailand[1] and Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, Hiroshima 739-8530, Japan[2]

Received 7 August 2017; accepted 1 October 2017
Available online xxx

***Chlorella* viruses or chloroviruses contain a gene that encodes an enzyme that catalyzes chitin synthesis. This gene is expressed early in viral infections to produce chitin on the outside of the *Chlorella* cell wall. Interestingly, chitin synthesis by microalgal *Chlorella* cells in combination with chloroviruses represents a unique eco-friendly process for converting solar energy and CO_2 into useful materials. However, during the final viral infection stage, the host cells are completely lysed, so chitin should be harvested before cells lyse. To increase chitin yields, slow-growing chlorovirus isolates were adopted and the viral replication process was modified with an inhibitor of DNA synthesis. The accumulation of chitin on the surface of *Chlorella* cells infected with one of nine chlorovirus isolates carrying the chitin synthase gene was compared with that of CVK2 (a standard virus)-infected cells. *Chlorella* cells infected with CVNF-1 (a slow-growing virus) accumulated chitin over the entire cell surface within 15 min post-infection (p.i.), and chitin continued to accumulate for up to 8 h p.i. before cells lysed. This was 2-fold longer than the chitin-accumulation period for cells infected with CVK2. The addition of aphidicolin delayed the progression of the virus replication cycle and extended the chitin-accumulation period of CVNF-1-infected cells to 12 h p.i. before cells lysed. Additionally, chitin production in the aphidicolin-treated CVNF-1-infected cells was approximately 6-fold higher than in CVK2-infected cells not treated with aphidicolin. Thus, chitin synthesis in a *Chlorella*-virus system may be prolonged by using slow-growing viral isolates treated with aphidicolin.**

[**Key words:** *Chlorella* cells; *Chlorella* viruses; Chloroviruses; Chitin material; Chitin synthesis; Aphidicolin]

Chitin (CH) is a β-(1,4)-linked homopolymer of *N*-acetyl-D-glucosamine (GlcNAc) present in the exoskeleton of arthropods, in the cell wall of fungi, and in various components of diverse invertebrates (1). It has diverse uses in industry, ranging from water purification to additives that thicken and stabilize food and pharmaceutical products. Industrially, CH has been extracted from crustacean shell wastes with chemical treatments (hydrochloric acid and sodium hydroxide) to remove associated minerals and proteins. However, the availability of crustacean shell wastes can be limited and is subject to seasonal supply. Moreover, the main issues concerning CH production are the risks posed by the chemical reagents used during the purification process, increases in the cost of raw materials, and environmental problems.

Under the current circumstances, a unique system to produce CH from culturable biomass such as algal cells based on solar energy conversion will be useful (2–4).

Chloroviruses or *Chlorella* viruses are large icosahedral, double-stranded DNA-containing viruses that infect certain strains of the unicellular green alga *Chlorella*. Chloroviruses encode genes involved in synthesizing polysaccharides such as CH or hyaluronan (HA), which is an alternating polymer composed of two monosaccharides, namely β-(1,4)-*N*-acetyl-D-glucosamine and β-(1,3)-D-glucuronic acid. Some chloroviruses contain genes encoding CH and HA synthases (CHS and HAS). These genes are expressed early during viral infections to produce HA or CH on the outside of the *Chlorella* cell wall (5). However, infected host cells are completely lysed in the final viral infection stage, resulting in degraded cellular polymers such as polysaccharides from the cell wall, proteins, lipids, and other components. Additionally, CH materials can be degraded by chitinases encoded by the chlorovirus itself (5). Thus, CH should be harvested before cells are lysed. Meanwhile, less CH is produced by the *Chlorella*-virus system than by the extraction of CH from crustacean shells. However, this system may provide a specific opportunity for basic and applied research of CH biosynthesis, especially considering it involves a potentially eco-friendly process using CO_2 and clean light energy.

The objective of this study was to isolate and investigate viruses from water samples collected from freshwater ponds in Higashi-Hiroshima, Japan and from natural water resources in Bangkok, Nakhon Pathom, Trat, Chonburi, Samut Sakhon, Lopburi, Pathumthani, and Nakhon Nayok provinces, Thailand. Moreover, the appearance and accumulation of polysaccharides on *Chlorella* cells were also examined, with trials conducted to prolong the accumulation of CH on virus-infected *Chlorella* cells.

* Corresponding author. Tel./fax: +81 082 424 7752.
E-mail address: tayamad@hiroshima-u.ac.jp (T. Yamada).

1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2017.10.002

Please cite this article in press as: Rakkhumkaew, N., et al., Chitin synthesis by *Chlorella* cells infected by chloroviruses: Enhancement by adopting a slow-growing virus and treatment with aphidicolin, J. Biosci. Bioeng., (2017), https://doi.org/10.1016/j.jbiosc.2017.10.002

MATERIALS AND METHODS

***Chlorella* and viruses** *Chlorella variabilis* strain NC64A cells (6,7) were cultured in modified Bold's basal medium (MBBM) as previously described (8). Chlorovirus PBCV-1 was a gift from Dr. J. L. Van Etten (University of Nebraska, Lincoln, NE, USA) and chlorovirus CVK2 (*Chlorella* virus Kyoto no. 2) was isolated from natural water resources in Kyoto, Japan using a plaque-forming assay (9,10). *Chlorella* cells were transferred to fresh MBBM medium every 2 weeks.

Detection and isolation of viruses Water samples were collected from freshwater ponds in Higashi-Hiroshima, Japan (34°24′04.5″N 132°42′45.3″E, 34°24′25.0″N 132°43′46.7″E, 34°24′27.9″N 132°42′18.6″E) and natural water resources in Bangkok (13°41′13.7″N 100°39′45.5″E), Nakhon Pathom (13°54′30.0″N 100°04′40.4″E), Trat (12°12′13.7″N 102°32′19.3″E), Chonburi (13°12′22.1″N 100°58′37.4″E), Samut Sakhon (13°38′25.1″N 100°18′04.0″E), Lopburi (15°12′08.0″N 100°55′09.4″E), Pathumthani (14°05′48.7″N 100°38′33.3″E), and Nakhon Nayok (14°06′06.0″N 100°59′10.7″E) provinces, Thailand. Water samples were filtered through a 0.45-μm pore membrane on site and, if necessary, concentrated by centrifugation at 15,000 ×*g* for 30 min. *Chlorella* viruses were detected using the plaque-forming assay described by Van Etten et al. (11), with *C. variabilis* strain NC64A as the host. A water sample was mixed with 200 μL host cells ($1–2 \times 10^8$ cells mL^{-1}). The mixture was added to 4 mL 0.7% soft-agar MBBM and poured onto a 1.5% MBBM agar plate, which was then incubated under fluorescent light at 25°C. Plaques were observed after 3 days of cultivation. Single plaques were transferred to plates containing a fresh algal lawn. Single virus strains were established by repeating this procedure several times.

Extraction of *Chlorella* virus DNA Virus particles collected by centrifugation (15,000 ×*g* for 30 min) were treated with 5 units mL^{-1} DNase I at 28°C for 1 h, after which they were washed and re-precipitated by centrifugation. The virus particles were then treated with proteinase K (at a final concentration of 1 mg mL^{-1} in 10 mM Tris buffer, pH 8.0, containing 1 mM EDTA and 1% *N*-lauroylsarcosine sodium salt) at 60°C for 1 h. The mixture was then subjected to a standard phenol extraction procedure (9).

Detection of the *CHS* gene A PCR was performed with DNA from 25 chlorovirus isolates used as templates to detect the *CHS* gene. The PBCV-1 and CVK2 DNA were used as negative and positive controls, respectively. The following primers were designed based on the CVK2 *CHS* sequence as described by Kawasaki et al. (2): forward primer, 5′-TGA GAA GGA AAC TGC ACA CGA ACT AC-3′; reverse primer, 5′-GGT AGG GAC GTA AAT ACC GTA ACA AGC-3′.

Analysis of CH accumulation on *Chlorella* cells infected with chloroviruses To examine CH production on the *Chlorella* cell surface, *C. variabilis* NC64A cells (10^7 cells mL^{-1}) were infected with chloroviruses at a multiplicity of infection of 10 and then incubated at 25°C in darkness. Infected cells were collected at various times post-infection (p.i.) and centrifuged at 3000 ×*g* for 10 min. The sediment was washed twice with cold phosphate-buffered saline (PBS) and then kept on ice for further analysis. The accumulation of CH on the surface of virus-infected cells was analyzed using biotinylated CH-binding proteins [wheat germ agglutinin-biotin for CH (J-Oil Mills, Inc., Tokyo, Japan)] in conjunction with avidin-Cy2 or -Cy3 (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Infected cells (1×10^7 cells mL^{-1}) were collected by centrifugation at 3000 ×*g* for 10 min, resuspended with 1 mL PBS, and then treated with 1.5 μg CH-binding protein and 5 μg avidin-Cy2 or -Cy3 on ice for 30 min. The cells were washed three times in PBS and incubated on ice before being examined under blue light (488 nm for Cy2) or green light (550 nm for Cy3) illumination using a BX60 fluorescence microscope (Olympus Corp., Tokyo, Japan).

***Chlorella* cells treated with aphidicolin** To prolong the accumulation of polysaccharides on *Chlorella* NC64A cells, aphidicolin (Sigma–Aldrich, St. Louis, MO, USA) diluted with DMSO (20 μg mL^{-1}) was added to *Chlorella* NC64 cells (10^7 cells mL^{-1}) 15 min prior to the chlorovirus infection at a multiplicity of infection of 10. Samples were collected at various times p.i. and centrifuged at 3000 ×*g* for 10 min. The sediment was washed twice with cold PBS. The accumulation of CH on virus-infected *Chlorella* cells was detected by microscopy using biotinylated CH-binding proteins in conjunction with avidin-Cy2 or -Cy3.

Quantification of CH accumulated on *Chlorella* cells Chlorovirus-infected *Chlorella* NC64A cells were collected at various times p.i. and CH was liberated by vortexing for 5 min with a Griner Vortex Mixer (at the highest power) (Griner Japan, Tokyo, Japan). Samples were centrifuged at 3000 ×*g* for 5 min and the pellet was discarded to remove the cells. The resulting supernatant was collected and centrifuged at 20,000 ×*g* for 30 min, after which the supernatant was discarded. Each pellet was suspended in 0.1 mL McIlvaine's buffer (12), pH 6.0, and treated with 5 μL purified *Streptomyces griseus* chitinase (5 mg mL^{-1} in PBS) (Sigma–Aldrich) for 24 h at 37°C to hydrolyze CH to GlcNAc. The samples were then heated to 99.9°C for about 60 s in a thermocycler, mixed gently, and incubated at 99.9°C for an additional 10 min. Immediately after cooling samples to room temperature, the GlcNAc content was quantified using the Morgan-Elson method (13). Briefly, 100 μL freshly diluted DMAB solution (Ehrlich's reagent; 10 g *p*-dimethylaminobenzaldehyde in 12.5 mL concentrated HCl and 87.5 mL glacial acetic acid, diluted 1:10 with glacial acetic acid) was added to the samples, which were then incubated at 37°C for 20 min. The abundance of GlcNAc was measured with a spectrophotometer at 585 nm. Standard curves were prepared using 0.01–0.1 mM GlcNAc stock solutions.

Electron microscopy Virus-infected *Chlorella* cells collected at various times p.i. were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 6.8) and post-fixed in 2% OsO_4 in the same buffer. After dehydrating in a series of ethanol concentrations (70–100%), cells were embedded in Spurr resin, and thin sections were cut using a Sorvall MT-1 ultramicrotome. The sections were stained with uranyl acetate and lead citrate and subsequently examined using a Hitachi H600A electron microscope (2).

RESULTS

Chlorovirus isolates Plaque-forming assays produced different-sized plaques on *Chlorella* lawns, and each plaque was further purified. After the purification, 11 algal-lytic viruses were obtained from freshwater samples collected in Higashi-Hiroshima, Japan. They formed variously sized plaques (0.1–3.0 mm) on NC64A lawns. The isolated viruses were designated as CVNF (*Chlorella* viruses New Field)-1 to CVNF-11. In contrast, plaques were not produced from water samples collected in Thailand, suggesting there is a lack of NC64A-infecting viruses in Thailand.

Detection of the *CHS* gene and analysis of CH accumulation on the surface of CVK2-infected cells To detect the *CHS* gene, a PCR was performed with genomic DNA isolated from 11 chlorovirus isolates as well as from two standard viruses, CVK2 and PBCV-1, which were used as the positive and negative controls, respectively. Six chlorovirus isolates were observed to carry the *CHS* gene (Fig. S1).

The accumulation of CH on the surface of CVK2-infected cells was observed as a standard pattern by fluorescence microscopy using a biotinylated CH-binding protein in conjunction with an avidin-Cy3 conjugate. Chitin began to accumulate on the host cell within 15 min p.i. and appeared as yellow spots on the cell surface (Fig. 1C). Most CVK2-infected cells developed uniform yellow fluorescent zones covering the entire cell surface by 2 h p.i. (Fig. 1D) and continuously accumulated CH until 4 h p.i. (Fig. 1E), after which they began to lyse (Fig. 1F). In contrast, CH could not be detected on uninfected (Fig. 1A) or PBCV-1-infected (Fig. 1B) cells using a biotinylated CH-binding protein in conjunction with an avidin-Cy3 conjugate. An electron microscopic observation revealed that fibrous substances (0.1 ± 0.05 μm thick, $n = 10$) covered the CVK2-infected *Chlorella* cells at 4 h p.i. (Fig. 1G).

For a comparison with CVK2-infected cells, the accumulation of CH on *Chlorella* cells infected with one of six chlorovirus isolates was investigated by fluorescence microscopy using a biotinylated CH-binding protein in conjunction with an avidin-Cy2 conjugate (for better resolution in comparative studies). Green fluorescence representing CH was observed covering most infected cells for up to 3.5–4.0 h p.i., after which the infected cells began to lyse. Interestingly, only CVNF-1-infected cells produced a strong CH signal for up to 8 h p.i. before lysing (Fig. 2A). Because of a longer CH-accumulation period that produced a thicker layer and brighter yellow fluorescence compared with that of the other CVNF isolates (data not shown), CVNF-1 was selected for further study.

Prolonged accumulation of CH on *Chlorella* cells induced by aphidicolin To prolong the accumulation of CH, *Chlorella* cells were treated with aphidicolin prior to the chlorovirus infection. Yanai-Balser et al. (14) reported that 20 μg mL^{-1} aphidicolin added to *Chlorella* NC64A cells completely inhibits viral DNA synthesis in 15 min.

Transcription of most late genes encoded by chloroviruses relies on the synthesis of viral DNA so the late-stages of infection to cell lysis were expected to be arrested. As described above, the

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