J. Microbiol. Biotechnol. (2002), **12**(4), 563–568

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Production of Recombinant Rotavirus Capsid Protein VP7 from Stably Transformed *Drosophila melanogaster* S2 Cells

PARK, JONG-HWA[1], KYUNG-HWA CHANG[1], YOUN-HYUNG LEE[1], HAE-YEONG KIM[2], JAI-MYUNG YANG[3], AND IN-SIK CHUNG[1,2]*

[1]*Department of Genetic Engineering,* [2]*Plant Metabolism Research Center and Graduate School of Biotechnology, Kyung Hee University, Suwon 449-701, Korea*
[3]*Department of Life Science, Sogang University, Seoul 121-742, Korea*

Received: January 22, 2002
Accepted: July 8, 2002

Abstract Stably transformed *Drosophila melanogaster* S2 cells producing recombinant VP7 were obtained, and recombinant VP7 expression was confirmed by Western blot analysis. The molecular weight of recombinant VP7 expressed in S2 cells was approximately 35.5 kDa, and 75% of the total VP7 produced was present in the medium. Recombinant VP7 contained *N*-linked glycosylated oligosaccharides. Aprotinin, leupeptin, and polyvinylpyrrolidone did not have any noticeable effect on recombinant VP7 production; however, DMSO and sodium butyrate increased its production by 120% and 60%, respectively.

Key words: *Drosophila melanogaster* S2 cell, rotavirus capsid protein VP7, DMSO, sodium butyrate

Rotavirus is a member of the Reoviridae family and causes acute gastroenteritis in young children and animals [14]. The outer layer of rotavirus is composed of the glycoprotein VP7, and it induces neutralizing antibodies that specify G serotype [21]. The VP7 gene contains an open reading frame of 326 amino acids which codes for two N-terminal hydrophobic sequences, H1 (residues 1-29) and H2 (residues 38-50), each of which can function as a signal peptide to direct the protein into the membrane of the endoplasmic reticulum [25]. The nascent VP7 is known as a 34-kDa glycoprotein that contains *N*-linked high-mannose oligosaccharide residues with one or more potential glycosylation sites [19].

VP7 genes of simian, bovine, and human rotavirus strains have been expressed in several systems to develop subunit or recombinant vaccines [7, 8, 10]. VP7 expressed in *E. coli* was found to be toxic to the cell [17], while a β-galactosidase-VP7 fusion protein induced a weak formation of neutralizing antibodies in mice [2]. Similarly, vaccinia virus constructs of the wild-type VP7 gene and a modified construct resulted in a partial secretion of VP7 [1], and induced only a small increase in neutralizing antibody when intradermally injected into rabbits. Rotavirus proteins expressed in a baculovirus-insect cell system have produced mixed results [5, 18].

In this study, the expression of cDNA coding human rotavirus VP7 and its glycosylation pattern from stably transformed *Drosophila melanogaster* S2 cells are described. The effects of aprotinin, leupeptin, polyvinylpyrrolidone (PVP), dimethyl sulfoxide (DMSO), and sodium butyrate on cell growth along with recombinant VP7 production were also investigated.

Materials and Methods

Cell Line, Plasmids, and Enzymes

Drosophila melanogaster Schneider 2 (S2) cells were grown at 27°C in T-25 (Nunc, Denmark) flasks in M3 medium (Shields and Sang M3 insect medium; Sigma, St. Louis, MI, U.S.A.) containing 10% IMS (insect medium supplement; Sigma). The plasmid pMT/BiP/V5-His (3.6 kb; Invitrogen, Carlsbad, CA, U.S.A.) contains a metallothionein promoter, a BiP signal sequence, a V5 epitope tag, and a polyhistidine region. The selection plasmid pCoHygro (Invitrogen), which contains the bacterial hygromycin B phosphotransferase gene under the control of the constitutive *Drosophila* Copia 5' LTR promoter, was used for stable transformation. The plasmid, pMBVP7, which contains human rotavirus VP7 encoding cDNA, was used. *E. coli* JM109 was used as the primary host for constructing and propagating plasmids. *E. coli* cells were routinely grown and maintained in LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl,

*Corresponding author
Phone: 82-31-201-2436; Fax: 82-31-202-9885;
E-mail: ischung@khu.ac.kr

pH 7.3) containing 50 mg/ml of ampicillin with agitation at 37°C. DNA restriction enzymes from Promega (Madison, WI, U.S.A.) or Takara (Shiga, Japan) were used according to the manufacturer's instructions.

Construction of Expression Plasmids

The human rotavirus VP7 (coding 51-326 amino acids) sequence was amplified from plasmid DNA, pMBVP7 [13], by PCR using oligonucleotide primers. The sense primer was 5'-AAAGATCTCAGAACTATGGAC-3' and the antisense primer was 5'- CTCGAGTACTCTATAATAAA-AC-3'. The amplified VP7 sequence was then inserted into the pGEM-T vector (Promega) to yield pGEM-T-VP7 and was confirmed by using a process of restriction enzyme mapping. PCR steps were performed in a Thermal Cycler (PE Biosystems, Foster City, CA, U.S.A.) by using PCR mix (Takara) in a 50 µl volume. pMT/BiP/VP7-V5-His (Fig. 1) was constructed by inserting a *Bgl*II-*Xho*I fragment of pGEM-T-VP7 between the *Bgl*II and *Xho*I sites of pMT/BiP/V5-His. The proper orientation and reading frame of the gene which were inserted in the recombinant plasmids of pMT/BiP/VP7-V5-His were confirmed by both restriction enzyme mapping and DNA sequencing.

Stable Transformation

Exponentially growing S2 cells were cotransfected with plasmids pMT/BiP/VP7-V5-His and pCoHygro (a ratio of 19:1) by using the lipofectin method as described elsewhere [20]. To prepare the transfection medium, plasmid DNA and lipofectin reagent (Gibco BRL, Grand Island, NY, U.S.A.) were separately diluted with IMS-free M3 medium, and then mixed together in a ratio of 1:5. The transfection medium was incubated at room temperature for 15 min and transferred into 6-well plates which were pre-seeded 2 h earlier with S2 cells in IMS-free M3 medium. After 24 h of incubation, the medium was changed to remove the lipofectin and the cells were incubated for 3 more days in M3 medium containing 10% IMS without hygromycin B selection. The cells were then centrifuged and resuspended in a selective M3 medium containing 10% IMS and 300 µg/ml hygromycin B. The selective medium was replaced every 5 days. Stably transformed polyclonal cell populations were isolated after being selected for 4 weeks of the selection process with hygromycin B. Hygromycin B was maintained routinely in the media at all times after selection.

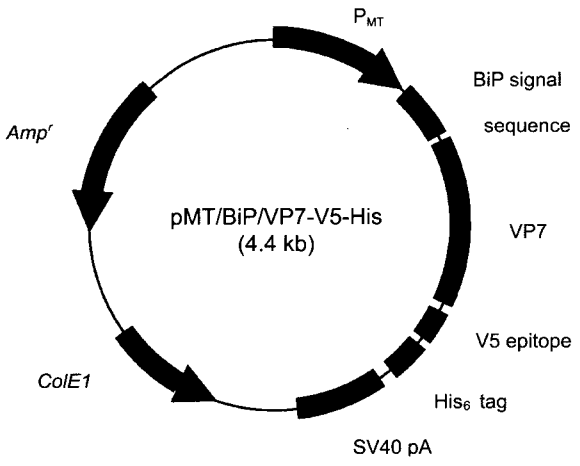

Fig. 1. Schematic representation of VP7 expression plasmid, pMT/BiP/VP7-V5-His.

Cell Culture and Analysis of Gene Expression

Stably transformed S2 cells expressing VP7 were grown at 27°C in T-25 flasks in 2–3 ml of M3 medium containing 10% IMS and 300 µg/ml hygromycin B. Unless otherwise specified, stably transformed S2 cells were cultured for 6 days in multiple T-25 flasks in order to analyze cell growth and VP7 expression. VP7 expression was induced by 0.5 mM $CuSO_4$ after the start of the run. To analyze the effects of protease inhibitors on gene expression, aprotinin (0–4 µg/ml), leupeptin (0–5 µg/ml) and PVP (0–1%) were added at the beginning of incubation. For determining what effects DMSO and sodium butyrate have on gene expression, DMSO and a concentrated sodium butyrate solution were added to the cultures immediately after $CuSO_4$ induction to obtain the desired concentrations, unless otherwise specified.

Cultures were centrifuged at 3,000 rpm for 5 min to separate the cells. The supernatant was used to identify extracellular recombinant proteins. The cell fraction was rocked for 1 h in a lysis buffer [50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 100 µg/ml of phenylmethylsulfonyl fluoride, 1 µg/ml of aprotinin, and 1% Triton X-100] and subjected to three freeze-thaw cycles between 10 min of −70°C freezer and 2 min of 37°C waterbath. After centrifuging the cell extracts at 14,000 rpm for 15 min to remove the cell debris, the supernatant was used to identify intracellular recombinant proteins.

VP7 expression was determined by densitometric scanning after SDS-PAGE and Western blot analyses. Protein concentrations were measured by Bradford protein assay kit (Bio-Rad, Hercules, CA, U.S.A.).

Treatment with Glycosidases

The recombinant VP7 was separated by SDS-PAGE and eluted from gel slices by incubating the slices in 2 ml of phosphate-buffered saline (PBS, pH 7.4) overnight at 4°C. After removing the gel slices from the PBS, the supernatant was centrifuged by using Centricon spin columns (Millipore, Bedford, MA, U.S.A). The VP7 fraction was incubated in PBS buffer with *N*-glycosidase F or *O*-glycosidase (Boehringer Mannheim, Mannheim, Germany) for 48 h at 37°C. Parallel incubation was performed under equivalent conditions in the absence of *N*-glycosidase F and *O*-glycosidase. These samples were analyzed by Western blot analysis.

Western Blot Analysis

Protein samples were analyzed by SDS-PAGE according to the Laemmlis method [16]. The electrophoresed proteins on the gel were transferred onto nitrocellulose, blocked with 3% BSA (bovine serum albumin), and incubated with a mouse anti-VP7 monoclonal antibody (1:50 v/v), and probed with a rabbit anti-mouse IgG alkaline phosphatase conjugate (1:1000 v/v). After washing, BM purple AP substrate solution (Boehringer Mannheim) was added and the reaction was quenched with distilled water.

RESULTS AND DISCUSSION

Recombinant VP7 Expression

Stably transformed S2 cells were obtained by using cotransfection of pMT/BiP/VP7-V5-His containing VP7 gene and pCoHygro. Recombinant VP7 expression at a 5 day post-induction with 0.5 mM $CuSO_4$ was analyzed by both SDS-PAGE and Western blot analyses (Fig. 2). Recombinant VP7 band was not clearly marked in SDS-PAGE, because the expression level was low. However, Western blot analysis with mouse anti-VP7 monoclonal antibodies showed that transformed S2 cell expressed a new protein with MW of about 35.5 kDa. Its molecular size was approximated with the predicted molecular weight of fused VP7 containing the C-terminal tag of V5 and His_6. The molecular weight of nascent VP7 protein is 34.6 kDa [9]. Recombinant VP7 was present in the intracellular and extracellular (medium) fractions of the transformed S2 cells. Densitometric scanning showed that secreted VP7 (VP7 present in the medium fraction) accounted for approximately 75% of the total VP7 production. Recombinant VP7 was not detected in either the cellular or the medium fractions of non-transfected S2 cells. This clearly indicates that the expression of VP7 was due to the integration of pMT/BiP/VP7-V5-His.

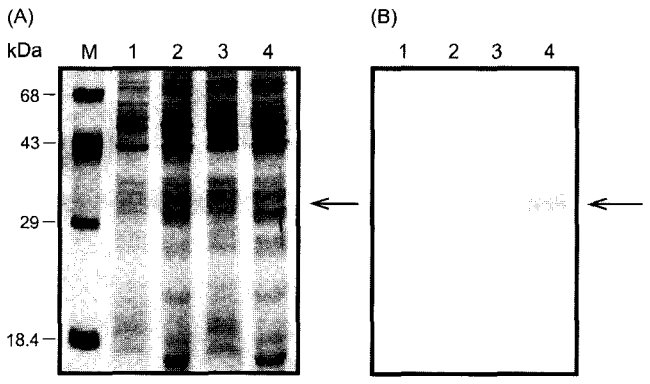

Fig. 2. SDS-PAGE (A) and Western blot (B) analyses of non-transformed and stably transformed S2 cells.
M) Molecular weight markers, 1) cellular fraction of non-transformed cells, 2) medium fraction of non-transformed cells, 3) cellular fraction of stably transformed S2 cells, and 4) medium fraction of stably transformed S2 cells. The arrow indicates the recombinant VP7 protein.

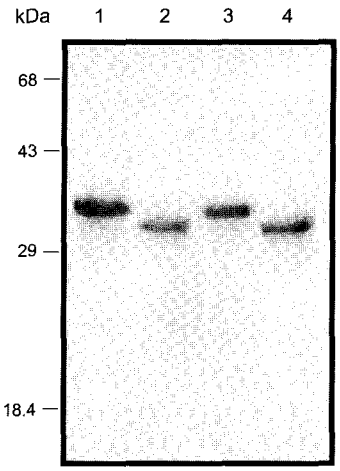

Fig. 3. Western blot analysis of recombinant VP7 fraction treated with glycosidases.
1, control; 2, VP7 treated with *N*-glycosidase F; 3, VP7 treated with *O*-glycosidase; 4, VP7 treated with *N*-glycosidase F and *O*-glycosidase.

Glycosidase Treatment

Recombinant VP7 protein was partially separated by using the gel-elution method [4]. The medium fraction of transformed S2 cells was separated by SDS-PAGE and eluted from gel slices by incubating the slices overnight at 4°C in 2 ml of PBS buffer. The supernatant was centrifuged by Centricon spin columns to concentrate the recombinant protein. In order to investigate whether the recombinant VP7 protein that was expressed in transformed S2 cells was glycosylated, partially purified VP7 was incubated with *N*-glycosidase F or *O*-glycosidase and analyzed by Western blot analysis (Fig. 3). VP7 treated with *N*-glycosidase F migrated as a band with MW 33 kDa. The 2.5 kDa difference between the molecular mass of the non-treated VP7 35.5 kDa and the treated 33 kDa was apparently due to glycosylation. In the case of *O*-glycosidase treatment, treated VP7 migrated as a band with the same molecular size as the untreated VP7. These results suggest that recombinant VP7 expressed in stably transformed S2 cells contained oligosaccharides that was formed by *N*-linked glycosylation.

Time-Course Changes During Cell Growth and Recombinant VP7 Expression

Stably transformed S2 cells were cultured for 7 days at an initial cell density of 5×10^6 and 10×10^6 cells/ml. Recombinant VP7 expression was induced through the addition of 0.5 mM $CuSO_4$ at day 0. The maximum cell density was 36×10^6 cells/ml at 3 days of incubation in the initial cell density of 10×10^6 cells/ml (Fig. 4). VP7 accumulation in the medium fraction reached the maximum level (~0.5 mg/l) at a 6-day post-induction.

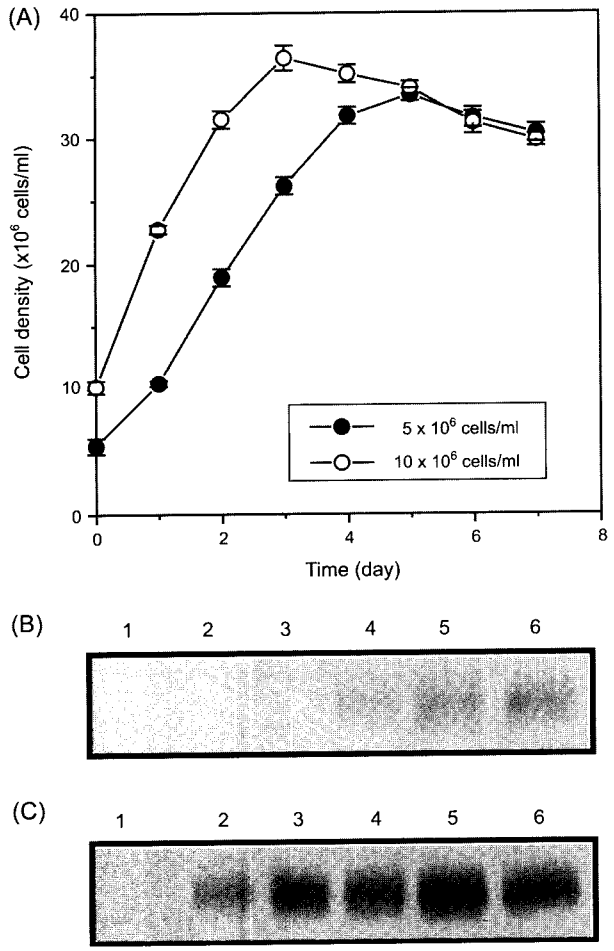

Fig. 4. Time-course of changes in cell growth and recombinant VP7 expression in T-flask cultures of stably transformed S2 cells.

In (A), the cell densities were plotted against the incubation time. In (B) and (C), recombinant VP7 expression in the medium fraction was confirmed by Western blot analysis. (B: 5×10^6 cells/ml; C: 10×10^6 cells/ml initial cell density). Lanes 1–6 represent the incubation period of 2–7 days, respectively. Error bars indicate standard deviation of the mean from three independent runs.

Effects of Medium Supplements on Recombinant VP7 Production

Aprotinin, leupeptin, and PVP have been used as protease inhibitors to increase the stability of expressed proteins in both baculovirus-insect and plant systems [6, 12, 15, 22]. Therefore, the effect of aprotinin, leupeptin, and PVP on recombinant VP7 production was examined. Transformed S2 cells were cultured for 6 days and recombinant VP7 expression was induced with 0.5 mM $CuSO_4$. Aprotinin, leupeptin, and PVP did not have any noticeable effect on cell growth and VP7 production (data not shown).

Dimethyl sulfoxide (DMSO) has been shown to be an effective permeabilizing agent which helps in the release of intracellular products from plant cells [23]. It is also known as a stabilizing agent for proteins [24] and can stabilize proteins against denaturation by forming hydrogen bonds with proton-donor groups on protein molecules [11].

The effect of DMSO on cell growth and VP7 production was examined. Transformed S2 cells were cultured for 6 days at an initial cell density of 10×10^6 cells/ml and induced by 0.5 mM $CuSO_4$ at day 0, and DMSO (2–8%) was added to the cultures at the beginning of incubation. After 6 days of incubation, the final cell densities and VP7 production were determined (Fig. 5). The addition of DMSO inhibited cell growth and increased the VP7 content in both cell extract and medium fractions. In 4% DMSO, the amounts of VP7 produced in cellular and medium fractions were maximal and they were 30% and 80% higher than the control, respectively.

Table 1 shows the effect of DMSO on cell growth and VP7 production as a function of time. A supplementation of DMSO at 2 or 4 days post-inoculation actually improved the final cell density and VP7 production, compared with a supplementation of DMSO at day 0. Maximal recombinant

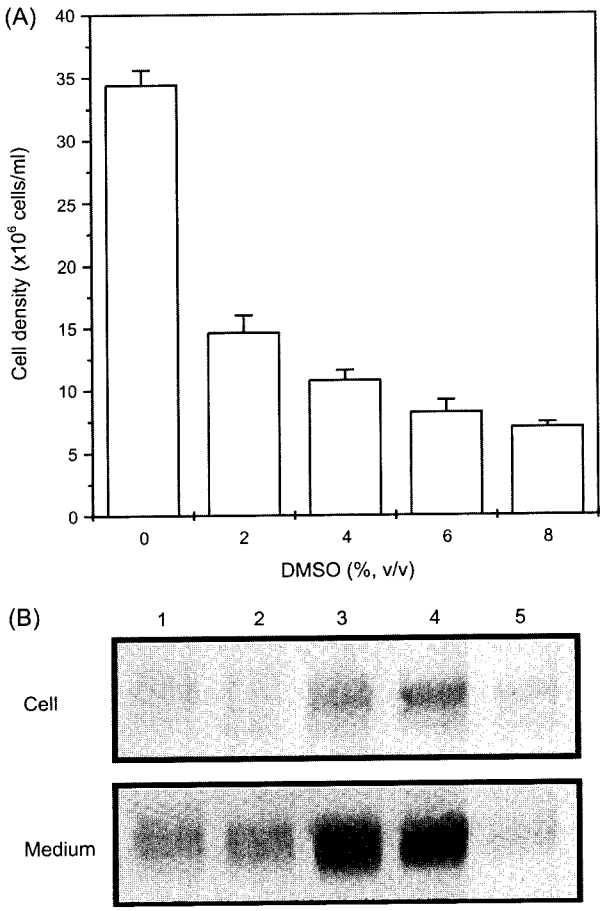

Fig. 5. Cell growth and recombinant VP7 production in DMSO supplemented culture of stably transformed S2 cells.

In (A), final cell densities are shown at different supplement levels of DMSO. In (B), recombinant VP7 expression was confirmed by Western blot analysis. Lanes 1–5 represent 0, 2, 4, 6, and 8% DMSO, respectively.

Table 1. Effect of the addition time of DMSO on cell growth and recombinant VP7 production[a].

Addition time (day)	DMSO (%)	Final cell density ($\times 10^6$ cells/ml)	Relative VP7 content	
			Cellular fraction	Medium fraction
2 days	0	33	100	100
	2	28	124	168
	4	22	151	199
	6	19	146	207
	8	16	113	115
4 days	0	34	100	100
	2	28	135	165
	4	26	175	224
	6	23	190	219
	8	21	150	103

[a]All data represent the average of duplicate experiments.

VP7 production was obtained at 4 days of 4–6% DMSO supplement. Recombinant VP7 produced in cellular and medium fractions were 90% and 120% higher than the control, respectively. These results showed that the supplementation of DMSO decreased the final cell density but increased the recombinant VP7 production in both cellular and medium fractions. The permeabilizing function of DMSO does not provide any explanation to the increased VP7 production in both cellular and medium fractions. The function of DMSO in recombinant VP7 production is still not fully understood, but it could be associated with the stabilization of the produced recombinant VP7 or the improvement of recombinant VP7 production by an unknown mechanism.

Stably transformed S2 cells were cultured for 6 days with and without sodium butyrate supplements. Sodium butyrate (2.5–30 mM) was added to the cultures at an initial cell density of 10×10^6 cells/ml. VP7 expression was induced with 0.5 mM $CuSO_4$ at the start of initial runs. After 6 days of incubation, the final cell densities and recombinant VP7 production were determined (Fig. 6). The supplementation of sodium butyrate decreased the final cell density: at 10 mM, the final cell density was decreased by 47%. Recombinant VP7 production reached to a maximum level with a supplementation of 5 mM sodium butyrate, and at this level of sodium butyrate, recombinant VP7 production was increased by 60% as compared to the control. At higher level of sodium butyrate than 5 mM, VP7 production was noticeably decreased, and this might be due to the decrease of cell growth. However, the addition of sodium butyrate did not affect the recombinant VP7 content in intracellular fraction (data not shown). The supplementation of sodium butyrate at 2 and 4 days post-inoculation increased the VP7 production by 36% and 21%, respectively, in the medium fraction, as compared to the supplementation of sodium butyrate at day 0. For other mammalian cells, sodium butyrate produces changes in the chromatin structure as a result of histones hyperacetylation which can be correlated with a modulation of gene expression [3].

The combined effect of DMSO and sodium butyrate on the recombinant VP7 production was investigated (data not shown). Sodium butyrate (5 mM) and DMSO (6%) were added to the culture at an initial cell density of 10×10^6 cells/ml. Recombinant VP7 production was determined after 6 days of incubation. The supplementations of both sodium butyrate and DMSO produced lower VP7 production, compared to the one which was supplemented with DMSO only.

In summary, the stable expression of the human rotavirus VP7 in *D. melanogaster* S2 cells was used to evaluate the *Drosophila* cell expression system. When expressed in *Drosophila* S2 cells under the influence of the *Drosophila* BiP protein signal sequence, the recombinant VP7 was found primarily in the medium fraction. The optimal production of recombinant VP7 was obtained from stably transformed S2 cells at 6 days after induction with 0.5 mM $CuSO_4$. The supplementations of DMSO and sodium butyrate increased the VP7 production up to ~1.1 mg/l, although the mechanism involved is unknown.

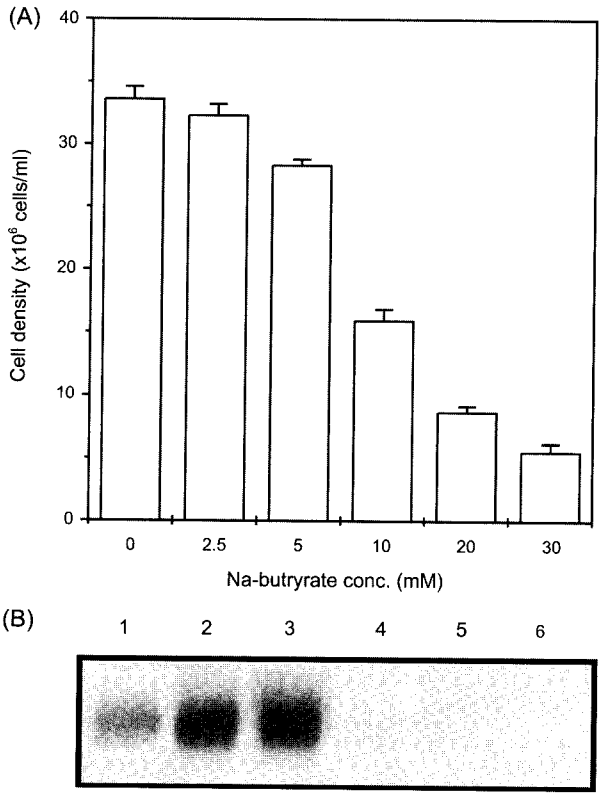

Fig. 6. Cell growth and recombinant VP7 production in sodium butyrate-supplemented culture of stably transformed S2 cells. In (A), final cell densities are shown for different supplement levels of sodium butyrate. In (B), recombinant VP7 expressed in medium fraction was confirmed by Western blot analysis. Lanes 1–6 represent 0, 2.5, 5, 10, 20, and 30 mM sodium butyrate, respectively.

Acknowledgments

This work was supported by grants from KOSEF (R01-1999-00174), the Korea Science and Engineering Foundation through the Plant Metabolism Research Center, Kyung Hee University, and the Brain Korea 21 project.

REFERENCES

1. Andrew, M. E., D. B. Boyle, B. E. H. Coupar, P. L. Whitfed, G. W. Both, and A. R. Bellamy. 1987. Vaccinia virus recombinants expressing the SA11 rotavirus VP7 glycoprotein gene induce serotype-specific neutralizing antibodies. *J. Virol.* **61:** 1054–1060.
2. Arias, C. F., T. Ballado, and M. Plebanski. 1986. Synthesis of the outer capsid glycoprotein of the simian rotavirus SA11 in *Escherichia coli*. *Gene* **47:** 211–219.
3. Arts, J., M. Lansink, J. Grimbergen, K. H. Toet, and T. Kooistra. 1995. Stimulation of tissue plasminogen activator gene expression by sodium butyrate and trichostatin A in human endothelial cells involves histone acetylation. *Biochem. J.* **310:** 171–176.
4. Bhown, A. S. and J. C. Bennett. 1983. High-sensitivity sequence analysis of proteins recovered from sodium dodecyl sulfate gels. *Meth. Enzymol.* **91:** 450–455.
5. Dharakul, T., M. Labble, J. Cohen, A. R. Bellamy, J. E. Street, E. R. Mackow, L. Flore, L. Rott, and H. B. Greeberg. 1991. Immunization with baculovirus-expressed recombinant rotavirus proteins VP1, VP4, VP6 and VP7 induces $CD8^+$ T lymphocytes that mediate clearance of chronic rotavirus infection in SCID mice. *J. Virol.* **65:** 5928–5932.
6. Dogan, B., H. S. Mason, L. Richter, J. B. Hunter, and M. L. Shuler. 2000. Process options in hepatitis B surface antigen extraction from transgenic potato. *Biotechnol. Prog.* **16:** 435–441.
7. Emslie, K. R., J. M. Miller, M. B. Slade, P. R. Dormitzer, H. B. Greenberg, and K. L. Williams. 1995. Expression of the rotavirus SA11 protein VP7 in the simple eukaryotic *Dictyostelium discoideum*. *J. Virol.* **69:** 1747–1754.
8. Estes, M. K., S. E. Crawford, M. E. Penaranda, B. L. Petrie, J. W. Burns, W. K. Chan, B. Ericson, G. E. Smith, and M. D. Summers. 1987. Synthesis and immunogenicity of the rotavirus major capsid antigen using a baculovirus expression system. *J. Virol.* **61:** 1488–1494.
9. Estes, M. K. 1990. Rotavirus and their replication *In*: *Virology*, pp. 1329–1347. 2nd ed. Raven Press, Ltd. New York, U.S.A.
10. Fiore, L., S. J. Dunn, B. Ridolfi, F. M. Ruggeri, and E. R. Mackow. 1995. Antigenicity, immunogenicity and passive protection induced by immunization of mice with baculovirus-expressed VP7 protein from rhesus rotavirus. *J. Gen. Virol.* **76:** 1981–1988.
11. Henderson, T. R., R. F. Henderson, and J. L. York. 1975. Effect of dimethyl sulfoxide on subunit proteins. *Ann. N. Y. Acad. Sci.* **243:** 38–53.
12. Hu, Y. C. and W. E. Bentley. 1999. Enhancing yield of infectious bursal disease virus structural proteins in baculovirus expression systems: Focus on media, protease inhibitors and dissolved oxygen. *Biotechnol. Prog.* **15:** 1065–1071.
13. Kang, D. K., K. W. Kim, P. H. Kim, S. Y. Seoung, Y. H. Kim, I. W. Kwon, S. Y. Jeong, E. Y. Choi, K. M. Lee, H. S. Kim, E. C. Kim, A. I. Joo, and J. M. Yang. 1998. cDNA cloning and expression of human rotavirus outer capsid protein VP7 in insect cells. *J. Microbiol. Biotechnol.* **8:** 369–377.
14. Kapikian, A. Z. and R. M. Chanock. 1990. Rotaviruses. *In*: *Virology*, pp. 1353–1404. 2nd ed. Raven Press, Ltd. New York, U.S.A.
15. LaCount, W., G. H. An, and J. M. Lee. 1997. The effect of polyvinylpyrrolidone (PVP) on the heavy chain monoclonal antibody from plant suspension cultures. *Biotechnol. Lett.* **19(1):** 93–96.
16. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227:** 680–685.
17. McCrane, M. A. and J. G. McCorquodale. 1987. Expression of a major bovine rotavirus neutralization antigen (VP7) in *Escherichia coli*. *Gene* **55:** 9–18.
18. McGonigal, T. D., M. C. Turon, K. Komar-Hartnett, S. E. Kister, and R. E. Smith. 1992. Expression of the gene coding for the major outer capsid protein of SA11 rotavirus in a baculovirus system. *Virus Res.* **23:** 135–150.
19. Offit, P. A. 1994. Rotaviruses; Immunological determinant of protection against infection and disease. *Adv. Virus Res.* **44:** 161–202.
20. Park, J. H., H. Y. Kim, K. H. Han, and I. S. Chung. 1999. Optimization of transfection conditions for expression of green fluorescent protein in *Drosophila melanogaster* S2 cells. *Enzyme Microb. Technol.* **25:** 558–563.
21. Paul, P. S. and Y. S. Lyoo. 1993. Immunogens of rotaviruses. *Vet. Microbiol.* **37:** 299–317.
22. Pyle, L. E., P. Banton, Y. Fujiwara, A. Mitchell, and A. Fidge. 1995. Secretion of biologically active human proapoliprotein A-1 on a baculovirus-insect cell system: Protection from degradation by protease inhibitors. *J. Lipid Research* **36:** 2355–2361.
23. Schmidt, A. J., J. M. Lee, and G. An. 1989. Media and environmental effects on phenylics production from Tobacco cell cultures. *Biotech. Bioeng.* **33:** 1437–1444.
24. Wahl, M. F., G. H. An, and J. M. Lee. 1995. Effects of dimethyl sulfoxide on heavy chain monoclonal antibody production from plant cell culture. *Biotechnol. Lett.* **17(5):** 463–468.
25. Whitfeld, P. L., C. Tyndall, S. C. Stirzaka, A. R. Bellamy, and G. W. Both. 1987. Location of signal sequence within the rotavirus SA11 glycoprotein VP7 which direct it to the endoplasmic reticulum. *Mol. Cell. Biol.* **7:** 2491–2497.