Thesis for the Degree of Master of Science

Hydroxyproline-containing collagen peptide derived from *Alaska pollack* skin inhibits HIV-1 infection

by

In-Seung Jang

Department of Chemistry

The graduate school

Pukyong National University

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PUKYONG NATIONAL UNIVERSITY
부경대학교

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Alaska pollack 유래 hydroxyproline 잔기 함유 콜라겐 펩타이드의 항 HIV-1 활성 연구

Advisor: Prof. Sun-Joo Park

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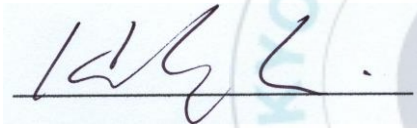

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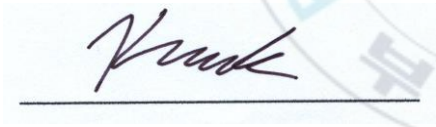

(Member) Prof. Min-Seok Kwak

(Member) Prof. Sun-Joo Park

August 21, 2015

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In-Seung Jang

Department of Chemistry, The Graduate School,

Pukyong National University

Abstract

Human Immunodeficiency Virus (HIV) is a lentivirus that causes the acquired immunodeficiency syndrome (AIDS). Anti-HIV therapy involving chemical drugs has improved the life quality of HIV/AIDS patients. However, emergence of HIV drug resistance, side effects and the necessity for long-term anti-HIV treatment are the main reasons for failure of anti-HIV therapy. Therefore, it is essential to develop novel anti-HIV therapeutics from natural resources. We here show that a hydroxyproline-containing marine collagen peptide (APHCP) inhibits HIV-1 infection to human T cells MT4. APHCP has been derived from *Alaska pollack* skin and previously reported to have potent antioxidant activity. The APHCP exhibited the inhibitory activity on HIV-1_{IIIB}-induced lytic effect (EC_{50}, 0.403 mg/ml, 459 μM), syncytia formation, viral p24 antigen production, and reverse transcriptase activity in MT4 cells. The anti-HIV activity was found to be specific against HIV-1 but not against HIV-2. Additionally, substitution of hydroxyproline residues in peptide by prolines resulted in decrease of its anti-HIV activity, suggesting that APHCP exhibits the anti-HIV-1 activity by scavenging intracellular reactive oxygen species generated during HIV-1 infection. These results suggest that marine peptide APHCP might be used as a drug candidate for the development of new generation therapeutic agents.

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List of Abbreviation

AIDS	Acquired immune deficiency syndrome
APHCP	*Alaska pollack* hydroxyproline containing collagen peptide
APPCP	*Alaska pollack* proline containing collagen peptide
Asp	Aspartic acid
AZT	Azidothymidine
BCIP	5-btomo-4-chloro-3`-indolyphosphate p-toluidine salt
BPB	Bromophenol blue
CCR5	C-C chemokine receptor type 5
CXCR4	C-X-C chemokine receptor type 4
CXCL12	C-X-C motif chemokine 12
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
gp120	Glycoprotein 120
gp41	Glycoprotein 41
HIV	Human immunodeficiency virus
MMP	Matrix metalloproteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NBT	Nitro-blue tetrazolium chloride
PVDF	Polyvinylidene fluoride
RPMI-1640	Roswell park memorial institute medium-1640
RT	Reverse transcriptase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
$TCID_{50}$	50% Tissue culture infective dos
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis

1. Introduction

The Human Immunodeficiency Virus (HIV) is a lentivirus that causes the Acquired Immune Deficiency Syndrome (AIDS). HIV infects vital cells in the human immune system such as T cells (specifically $CD4^+$ T cells), macrophages, and dendritic cells and leads to low levels of $CD4^+$ T cells. When $CD4^+$ T cells decline below a critical level, cell-mediated immunity is lost, and the body becomes progressively more susceptible to life-threatening opportunistic infection. Without treatment, average survival time after infection with HIV is estimated to be 9 to 11 years. AIDS occurs when a person with HIV infection has a $CD4^+$ cell count below 200 cells per μl (Kent, 2001).

AIDS was firstly observed in 1981 in USA. After 2 years, Luc Montagnier group of institute Pasteur in France and Robert Gallo group of national institutes of health in USA independently discovered the presence of virus causing AIDS (Gallo et al., 1983; Montagnier et al., 1983). According to the researchers who isolated virus, Montagnier group named as LAV (Lymphadenopathy-associated virus), Gallo group named as HTLV-III (human T-cell lymphotropic virus type III), and Levy group called as ARV (AIDS-related virus). However, all virus forms are similar to the lentivirus. It was finally named with HIV in 1986 (Aldrich, 2001). In the same year, Montagnier group also found new type of HIV in West Africa and named HIV-2 to distinguish it from HIV-1. AIDS also occurs in people infected with HIV-2, although it takes a longer time to develop AIDS and has low prevalence rate (Table 1).

Table 1. Classification of HIV

Species	Virulence	Infectivity	Prevalence	Inferred origin
HIV-1	High	High	Global	Common Chimpanzee
HIV-2	Lower than 1	Low	West Africa	Sooty Mangabey

HIV is transmitted by three main routes, sexual contact, exposure to infected fluids or tissues by syringe or something else, and vertical transmission from mother to child (Luiza, 2015). After transmission, HIV infects the immune cells. HIV has the glycoproteins called gp120 and gp41. The gp120 is attached to CD4 receptor and the CXCR4 co-receptor of T cells or the CCR5 co-receptor of dendritic cells. Membrane fusion between virus and host cells is occurred by the gp41. In host cells, a viral enzyme called reverse transcriptase copies HIV RNA genome into double-stranded DNA. These DNA inserts into host DNA by other viral enzyme, integrase (Korovina, 2014). Once integrated into the host cell DNA, HIV begins to use the machinery of the host cell to make RNA and proteins. Recombinant RNA and proteins move to the surface of host cell. In the surface, RNA and proteins assemble for budding. After the HIV protease cuts the long protein chains to form the immature virus, the immature virus can infect other immune cell (Munir et al., 2013; Gilmore et al., 2013; Weiss et al., 2011) (Fig. 1).

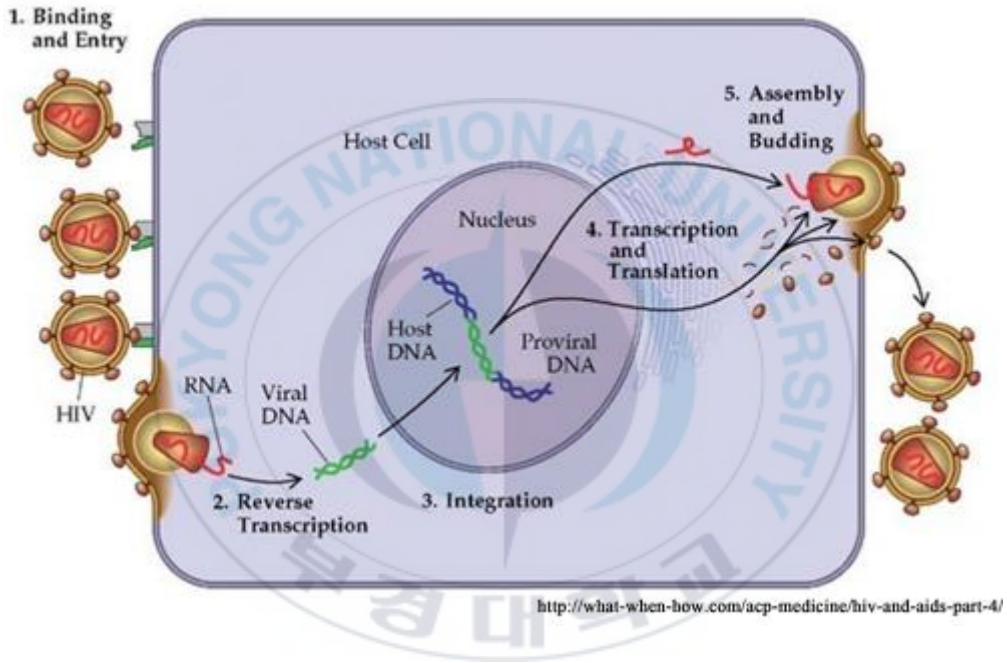

Figure 1. Major steps in the life cycle of HIV

After first discovery, the number of AIDS patients is increasing steadily, 35 million people infected worldwide by 2013. In 2013, 2.1 million people newly infected with HIV and 1.5 million people died due to AIDS. In Korea, first infected patient was discovered at 1985 and 8,662 people infected by 2013(Kasedde et al., 2015). In accordance with the increase in HIV patients, many researchers have studied consistently to develop HIV medicine. In 1987, Zidovudine (trade name AZT) was first approved from USA government as HIV medicine. After that, many medicines have been developed. Plerixafor (trade name Mozobil) that synthesized by Fabbrizzi et al. (1987) could have a potential use in the treatment of HIV because of its specific role in the blocking of CXCR4. However, use of these medicines and development of this indication have been terminated because of severe side effects such as lacking oral availability and cardiac disturbances. In addition, as HIV multiplies, it easily mutates and produces variations of itself. Variations of HIV that develop while a person is taking HIV medicines lead to drug-resistant strains of HIV. HIV medicines that previously controlled the person's HIV were not effective against the new, drug-resistant HIV. Therefore, in recent, to minimize the resistance and to delay the progression of HIV to AIDS, multiple drugs that act on different viral target are usually used in combination. Use of these drugs in combination is termed as 'highly active antiretroviral therapy (HAART)' or 'cocktail therapy' (Ceccherini-Silberstein, 2011). Patients receiving combination therapy have reported decreased viral loads, increased CD4 counts, and increased T-cell counts. The anti-retroviral therapy has extended and enhanced the quality of life of HIV-infected patients. However, HAART is far from perfect treatment for HIV and access to such treatment remains a major concern in most parts of the world, particularly in the developing countries. In the developing world, treatment for opportunistic infection is almost unavailable, and low cost treatments was desperately

necessary.

In this regard, natural bioactive compounds have been attracted the attention as great sources for the development of new generation anti-HIV therapeutics which are more effective with fewer side-effects and low cost. In particular, marine resources would be the leading sources of anti-HIV natural products. Marine organisms produce very different kinds of substances because they are living in a very unique, exigent, and aggressive environment. Recently, a great deal of interest has been expressed regarding marine-derived anti-HIV agents. Several studies have reported that marine peptide can be used as anti-HIV agents in functional foods or nutraceuticals and pharmaceuticals due to their therapeutic potential in the treatment or prevention of infectious diseases (Lee and Mruyama, 1998; Plaza et al., 2007 and 2009; Zampella et al., 2008; Oku et al., 2004; Andjelic et al., 2008, Ngo et al., 2012). The activity of marine-derived peptides is based on the amino acid composition and sequence.

In light of the need for new anti-HIV marine peptides, we here examined anti-HIV activity of peptides derived from several marine organisms (Table 2). In particular, we focused on anti-HIV activity of marine peptides reported to have antioxidant activity. HIV infection has been thought to lead to augmented oxidative stress which in turn leads to faster development of HIV disease, although its molecular mechanism remains unclear (Peterhans et al., 1988; Baeuerle and Baltimore, 1988; Jarstrand and Akerlund, 1994; Pace and Leaf, 1995; Sepulveda and Watson, 2002; Singh and Pai, 2015). HIV-infected and AIDS patients have exhibited elevated serum levels of hydroperoxides and malondialdehyde, which are the byproducts of lipid peroxidation (Meyer et al., 1994; Favier et al., 1994; Revillard et al., 1992; Sonnerborg et al., 1988). Excessive production of reactive oxygen species (ROS) was found in the neutrophiles of HIV-

infected patients, whose antioxidant defense systems undergo dramatic changes (Jarstrand and Akerlund, 1994). Recent work has shown that HIV-1 is also able to induce ROS production in astrocytes and microglia (Carrol-Anzinger et al., 2007; Reddy et al., 2012). Therefore, oxidative stress might lead to evolution of HIV disease through various routes such as devastation of immune function, enhancement of HIV replication, and augmentation of apoptosis. Antioxidants may have a significant role in the treatment of HIV/AIDS. The challenge with antioxidant marine peptides for the prevention and treatment of HIV may provide a valuable clue for development of new HIV drugs.

2. Material and methods

2.1. Materials and chemicals

Azidothymidine (AZT), zidovudine, and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Sigma Aldrich (St. Louis, US). Marine peptides were synthesized based on the amino acid sequence previously reported (Kim et al., 1984 and 2007; Medis et al., 2005; Qian et al., 2008; Ryu et al., 2010; Ko et al., 2012) (Peptron, Daejeon, Republic of Korea). DMSO were obtained from Amresco (Solon, USA). Specific antibodies for western blot were purchased from R&D systems (NE Minneapolis, USA) and Santa Cruz Biotechnology (Dallas, USA).

2.2. Cell culture

MT-4 cell lines, H9/HIV-1_{IIIB} and H9/HIV-2_{ROD} cell lines were obtained through NIH AIDS Reagent Program (Germantown, USA). MT-4 and H9/HIV-1_{IIIB} and H9/HIV-2_{ROD} cell lines were grown in RPMI-1640 (Thermo Scientific, USA) supplemented with 10% heat-activated FBS (Thermo Scientific, USA), 50 μg of streptomycin per ml and 50 U of phenicillin per ml (PAA, USA) in 5% CO_2-containing air at 37 ^oC. Cells were cultured at 2 ~ 4 days intervals and maintained 5 x 10^5 ~ 1 x 10^6 cells/ml.

2.3. Virus

HIV-1_{IIIB} and HIV-2_{ROD} were obtained from supernatants of H9/HIV-1_{IIIB} and H9/HIV-2_{ROD} cell lines. The virus were stored at -80 ^oC until use. Virus titer was determined by measuring the number of p24 antigen when HIV-1_{IIIB} infected to MT-4 cells. Virus titer was expressed as $TCID_{50}$

2.4. Cell viability assay

The 50% cytotoxic concentration of AZT and APHCP were determined by MTT assay. For the assay, MT-4 cells were seeded in a 96-well plate at 2 x 10^4 cells/well with RPMI-1640 medium containing 10% FBS. After 24 h of incubation, the cells were treated with AZT and APHCP of 0 mg/ml to 0.75 mg/ml concentration and incubated for 24 h at 37 ºC. 24 h later, every well was changed fresh RPMI-1640 medium with 10% FBS. After 84 h, 20 μl of MTT solution (Final concentration : 0.5 mg/ml) was added to each well and the plate was incubated for 4 h at 37 ºC. Lastly, 200 μl of DMSO was added to dissolve the purple formazan. The amount of formazan was determined by measuring the absorbance at 595 nm using microplate reader (Filter Max 5, Molecular Devices). Cell viability was determined compared to that of untreated MT-4 cells.

2.5. Inhibition of lytic effect of HIV-1

In order to determine the anti-HIV activity of APHCP on HIV infected MT-4 cells, MTT assay was used. MT-4 cells were washed and resuspended with fresh RPMI-1640 medium, and seeded in duplicate to the wells of a 96-well plate with 2 x 10^4cells/well. After 24 h, stock virus of HIV-1_{IIIB} and HIV-2_{ROD} were added to the each well at 25 $TCID_{50}$ with the dilutions of APHCP. The plate was incubated for 84 h at 37 ºC with 5% CO_2. Cell viability was determined by MTT assay as described previously (Artan, 2008).

2.6. p24 antigen assay

2 x 10^4 cells of MT-4 cells were seeded in plate. After 1 day, MT-4 cells were treated with APHCP and infected with 25 $TCID_{50}$ of HIV-1_{IIIB} and HIV-2_{ROD}. The plate was incubated for 84 h. The supernatant in wells was harvested by centrifugation. In order to determine the amount of HIV, Lenti-X p24 rapid titer kit was used according to the protocol (Clone tech, USA).

2.7. Reverse Transcriptase activity assay

The activity of HIV-1 reverse transcriptase in the virus supernatant was determined by using a reverse transcriptase assay kit (Roche, Germany) according to the protocol. Briefly, reaction mixture containing poly(A) x $oligo(dT)_{15}$ was added to the virus supernatant and incubate for 4 h at 37 ºC. 200 µl of anti-DIG-POD and ABTS was added stage by stage. The virus supernatant was incubated at room temperature until color development is sufficient for detection. The absorbance of the virus supernatant was measured by using micro plate reader at 405 nm.

2.8. Syncytia formation analysis

Inhibition effect of APHCH on syncytia formation was determined by microscope. 2 x 10^4 MT-4 cells were seeded in 96 well. After 24 h of incubation, MT-4 cells infected with 10 µl of stock supernatant of HIV-1_{IIIB} diluted at 25 $TCID_{50}$ with/without APHCP. The plate was incubated 3 days and the number of syncytia formation was counted microscopically.

2.9. Western blot analysis

MT-4 cells were seeded at 2 x 10^4cells/well in 80 µl of fresh medium and incubated for 1 day at 37 °C with 5% CO^2. MT-4 cells was infected HIV-1_{IIIB} stock supernatant with/without APHCP. After 84 h incubated, MT-4 cells were pelleted at 1000 rpm for 5 min and separated from supernatant. MT-4 cell pellets were harvested and solubilized in 2x SDS sample buffer containing 100 mM DTT, 100 mM Tris-HCl, 0.4% BPB, and 20% glycerol. Equal volume of virus lysate was loaded onto SDS-PAGE gel. Separated proteins were transferred to PVDF membrane (Millipore Corp. USA). The membranes were blocked for 1 h with 1% BSA in TBS-T (10 mM Tris-HCl, 150 mM NaCl [pH 7.5] containing 0.1% Tween-20), and incubated 1 h with primary antibodies against p24 and Actin. The membranes were then washed with TBS-T and incubated for 30 min with the appropriate secondary antibody conjugated to alkaline phosphatase. The respective protein bands were detected by BCIP/NBT Alkaline phosphatase reaction.

3. Results

3.1. Anti-HIV-1 activity of peptides with antioxidant activity

Recent works have shown that HIV-1 induces reactive oxygen species (ROS) production (Ardavin et al., 1997; Wang et al., 2011; Pyo et al., 2008). We here examined the anti-HIV activity of several marine peptides that reported to have antioxidant activity (Table 2). MT-4 cells were seeded to 96-well plate and treated with peptides of 0.5 mg/ml concentration before infection with HIV-1_{IIIB}. After incubation for 84 h, the viability of MT-4 cells was measured by MTT assay. Figure 1 shows the relative cell viability compared to those of peptide-untreated and HIV-1_{IIIB}-treated control cells. Among nine peptides, only P2 peptide was found to have anti-HIV activity. P2 peptide is derived from protease E hydrolysate of *Alaska pollack* skin (Kim et al., 1984). The amino acid sequences of P2 peptide is Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly and its chemical structure was shown in Fig. 2. P2 is named to APHCP (*Alaska Pollack* skin Hydroxyproline containing Collagen Peptide).

Table 2. List of marine peptides with antioxidant activity

Peptide No.	Amino acid sequence	Source	Reference
P1	LEDPFDKDDWDNWKS	*Hippocampus kuda Bleeler*	Ryu et al., 2010
P2	GPHypGPHypGPHypG	*Alaska pollack*	Kim et al., 1984
P3	GSTVPERTHPACPDFN	*Johnius belegnerii hoki*	Kim et al., 2007
P4	LEELEEELEGCE	*bullfrog*	Qian et al., 2008
P5	NGPLEAGQPGER	*Dosidicus gigas*	Mendis et al., 2005
P6	AHIII	*S. clava*	Ko et al., 2012
P7	MLLCS	*S. plicata*	
P8	LNGDVW	*C. ellipsoidea*	Ko et al., 2012
P9	LLLGD	*S. plicata*	

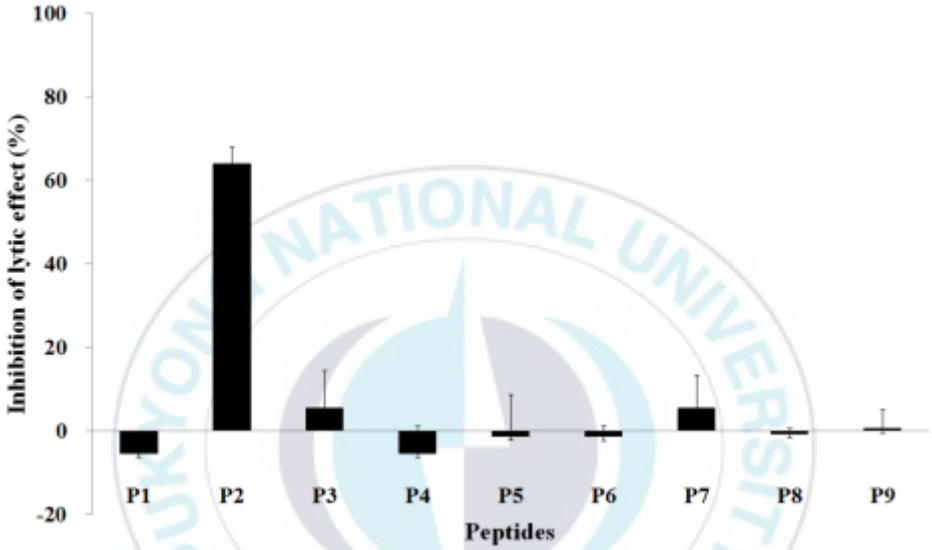

Figure 2. Anti-HIV-1 activity of marine peptides. Inhibitory lytic effect of antioxidant peptides were measured by MTT assay. MT-4 cells were infected with HIV-1_{IIIB} of 25 $TCID_{50}$ in the presence or absence of peptides. Inhibitory effect of peptides against HIV-1_{IIIB} infection were determined by MTT assay.

A

Gly - Pro - Hyp - Gly - Pro - Hyp - Gly - Pro - Hyp - Gly

B

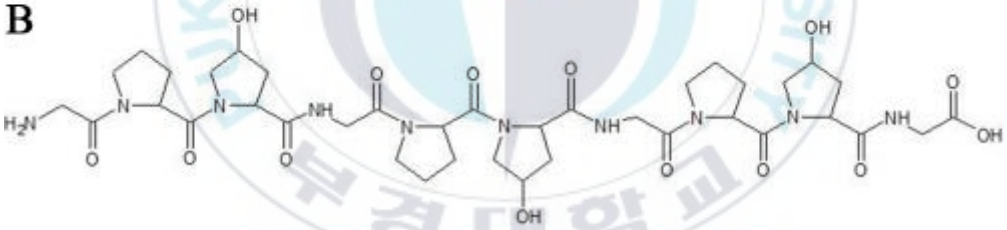

Figure 3. Amino acid sequence (A) and chemical structure (B) of APHCP

3.2. Non cytotoxicity of APHCP

The cell cytotoxicity of APHCP is first examined in human MT-4 T cells before examing its potential anti-HIV activity. MT-4 cells were treated with APHCP of 0 ~ 0.75 mg/ml concentration for 84 h. The viability of MT-4 cells was measured by MTT assay. As shown in Fig. 3, APHCP did not affect the viability of MT-4 cells at concentration below 0.75 mg/ml.

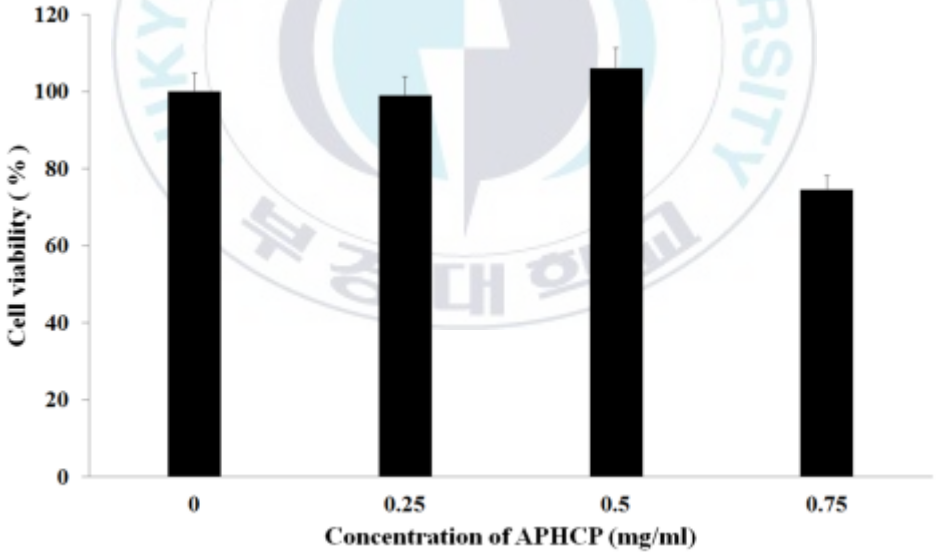

Figure 4. Effect of APHCP on viability of MT-4 cells. MT-4 cells were treated with 0 ~ 0.75 mg/ml APHCP for 84 h and the viability of MT-4 cells were measured by MTT assay.

3.3. Protective effect of APHCP on HIV-1-induced lytic effect

When infected virus budded from host cells, the cells were lysed and destroyed. This phenomenon is lytic effect by HIV virus. The protective activity of APHCP on HIV-1_{IIIB}-induced lytic effect was examined by MTT assay. Since MTT selectively reacts with live cells, if APHCP has inhibitory effect against HIV-1-induced cell lysis, MTT value should be increased in cells treated with APHCP. Cell lysis by HIV-1_{IIIB} infection was significantly decreased by treatment with APHCP (Fig. 4). EC_{50} of APHCP against anti-HIV-1_{IIIB} infection was assessed by 0.403 mg/ml, 459 μM.

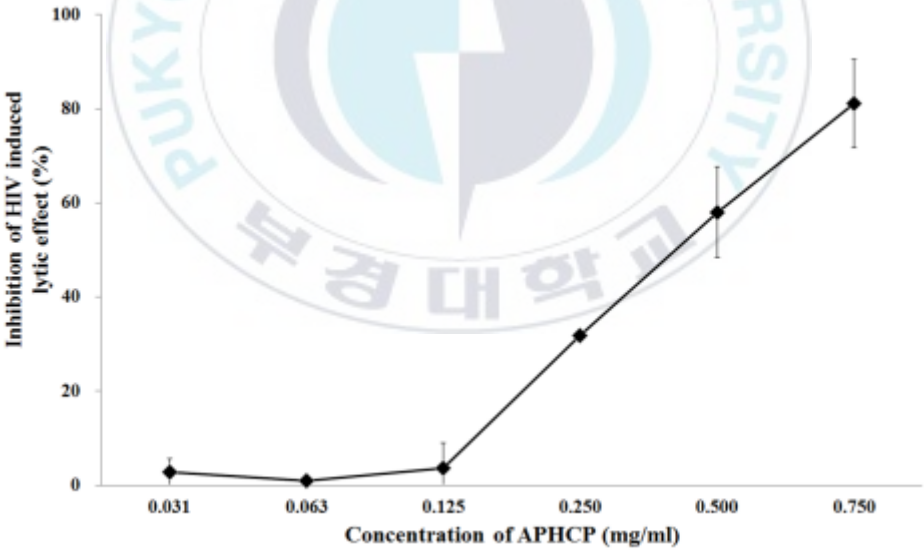

Figure 5. Inhibitory lytic effect of APHCP. MT-4 cells were infected with HIV-1_{IIIB} at 25 $TCID_{50}$ with 0 ~ 0.75 mg/ml APHCP. After treatment, MT-4 cells were incubated for 84 h. Inhibition lytic effect of APHCP was determined by MTT assay.

3.4. Effect of APHCP on p24 antigen production

The HIV gag gene encodes p24, a lentiviral capsid protein. Since p24 is indispensable for subsequent reproduction of HIV in HIV-1 infected cells, it is an essential element for HIV infection. p24 is detected from early stage of HIV infection. Inhibitory effect of APHCP on p24 production was detected with p24 antigen capture ELISA. p24 is assessed in a sandwich ELISA format using the biotinylated anti-p24 secondary antibody. p24 antigen production of more than 90% was suppressed in cells treated with 0.5 mg/ml APHCP (Fig. 5). The relative value to those of peptide-untreated and HIV-1_{IIIB}-treated control was exhibited as a percent. EC_{50} of APHCP was calculated by 0.356 mg/ml, 405 μM.

Inhibitory effect of APHCP on HIV-1_{IIIB}-induced p24 protein production was also examined by western blot analysis with p24 primary antibody. p24 protein is produced by HIV-1_{IIIB} infection to MT-4 cells and the increased p24 is decreased by APHCP treatment.

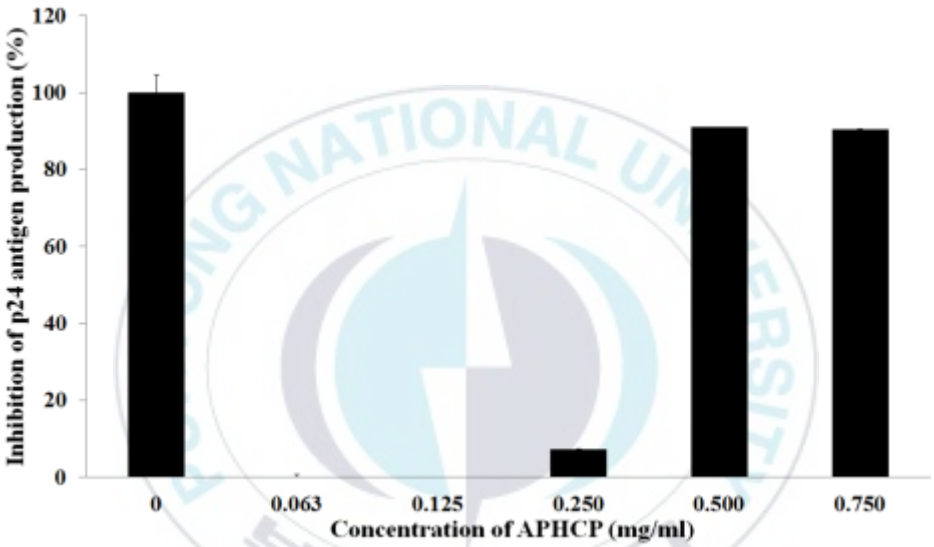

Figure 6. Inhibitory effect of APHCP on HIV-1 p24 production. MT-4 cells were infected with HIV-1_{IIIB} of 25 $TCID_{50}$. The amount of p24 antigen production was determined by p24 antigen capture ELISA. Peptide-untreated MT-4 cells were used as positive control.

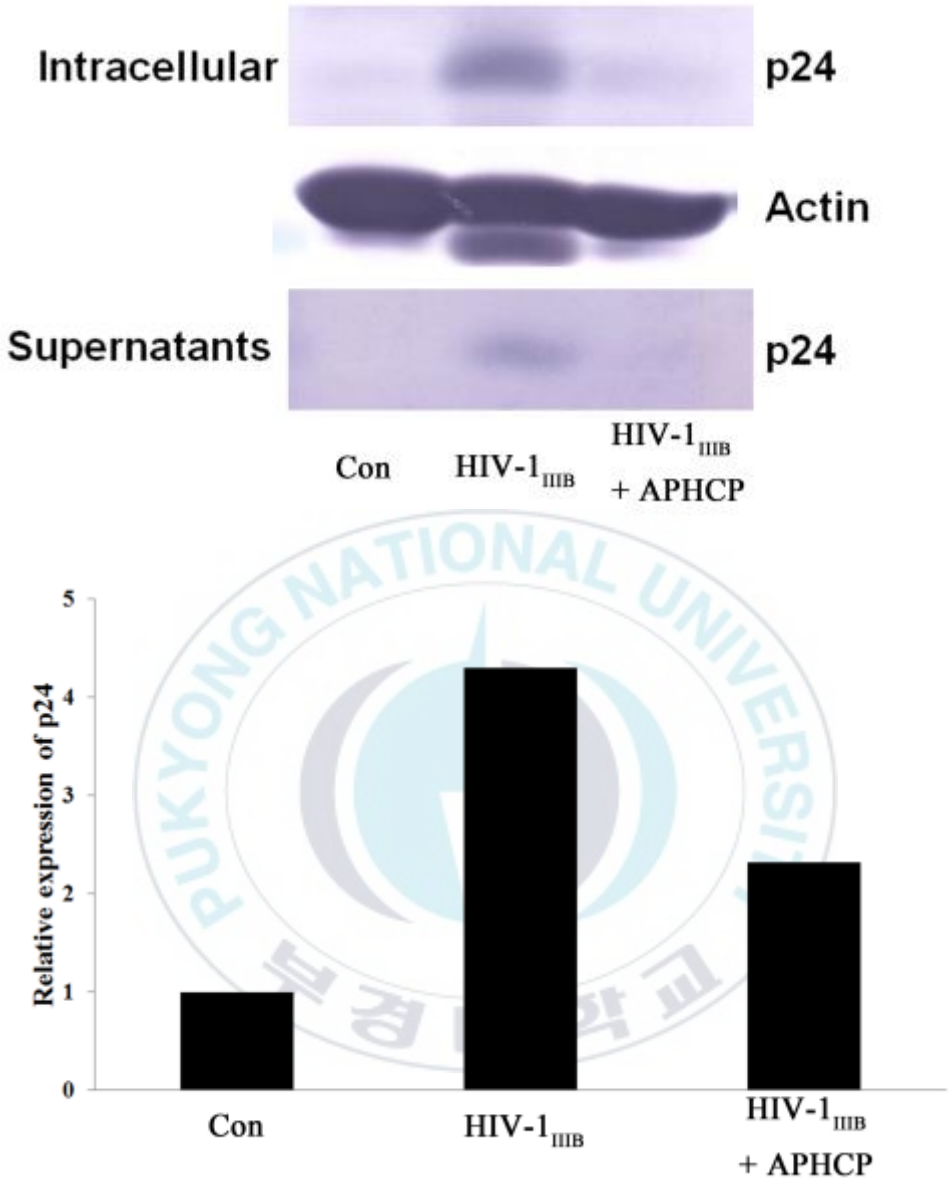

Figure 7. Inhibitory effect of APHCP on viral p24 protein production in HIV-1_{IIIB} infected MT-4 cells. (Up) Intracellular and secreted p24 proteins were analyzed by western blot analysis. (Down) Areas and intensities of protein bands were measured by densitometry and expressed as a percentage of p24 expression compared to protein level of uninfected cells.

3.5. Effect of APHCP on reverse transcriptase activity

Viral RNA is transmitted to DNA by the reverse transcriptase (RT) in infected host cells. Anti-HIV activity of APHCP is also examined on RT activity. As shown in Fig. 8, APHCP effectively inhibited HIV-1_{IIIB}-induced RT activation. The inhibitory potential of APHCP was similar to the results found in p24 antigen capture ELISA assay. The RT activity was completely suppressed by cells treatment with 0.5 mg/ml APHCP. In concentration of 0.5 mg/ml APHCP, the RT activity by HIV-1_{IIIB} infection was completely inhibited to approximately 95% of untreated control. EC_{50} was assessed by 0.327 mg/ml, 0.374 μM.

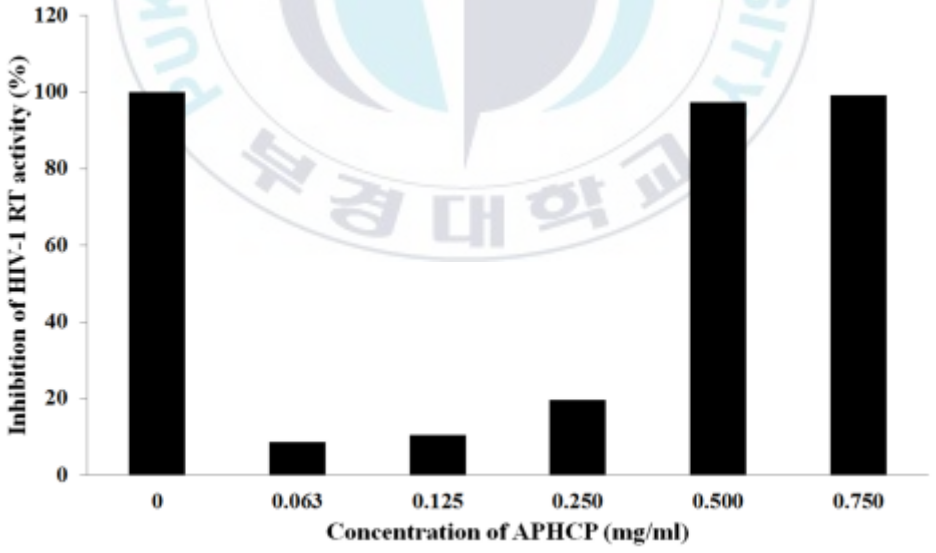

Figure 8. Inhibitory effect of APHCP on reverse transcriptase activity. The effect of APHCP on HIV-1 reverse transcriptase activity inhibition was determined by colorimetric RT activity assay. Uninfected MT-4 cells were used as positive control.

3.6. Effect of APHCP on syncytia formation

Syncytia formation between virus infected cells and uninfected cells is another feature representing HIV infection. Fused cell is destroyed within a few days. gp120 in virus-infected cells is responsible for cell fusion to uninfected $CD4^+$ T cells. APHCP-treated MT-4 cells are similar to that of uninfected control cells, whereas HIV-1_{IIIB}-infected MT-4 cells fused with other cells and formed syncytia. This result indicates that APHCP inhibits the HIV-1_{IIIB}-induced syncytia formation (Fig. 9).

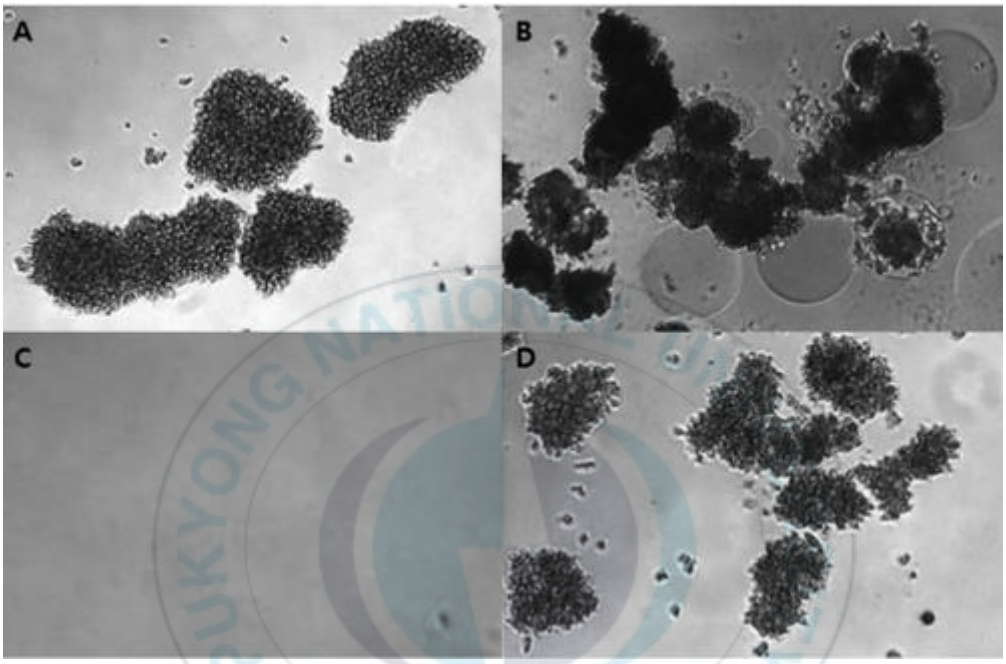

Figure 9. Microscope images of MT-4 cells infected with HIV-1_{IIIB}. MT-4 cells were infected with HIV-1_{IIIB} of 25 $TCID_{50}$ in the presence or absence of APHCP (0.75 mg/ml) for 24 h. After 24 h, all wells were changed with fresh medium, then furthermore incubated for 3 days. Syncytia formation was detected using an inverted microscope (100X). (A) Uninfected cell control, (B) HIV-1_{IIIB}-infected cells, (C) HIV-1_{IIIB} virus, and (D) HIV-1_{IIIB} infected cells with APHCP.

3.7. Amino acid substitution of APHCP

To examine essential residue responsible for anti-HIV activity of APHCP, we synthesized APPCP peptide that hydroxyproline residues in the APHCP are replaced by prolines because that hydroxyl group has been know to target free radical reactive oxygen species (Jung, 2013; Dugas, 2000; Iglesias, 1999). Anti-HIV activity of APPCP was examined by measuring HIV-1_{IIIB}-induced lytic effect. As the result, APPCP did not show the anti-HIV activity against HIV-1_{IIIB} infection (Fig. 10), indicating that hydroxyl group of hydroxyproline is essential for anti-HIV activity of APHCP.

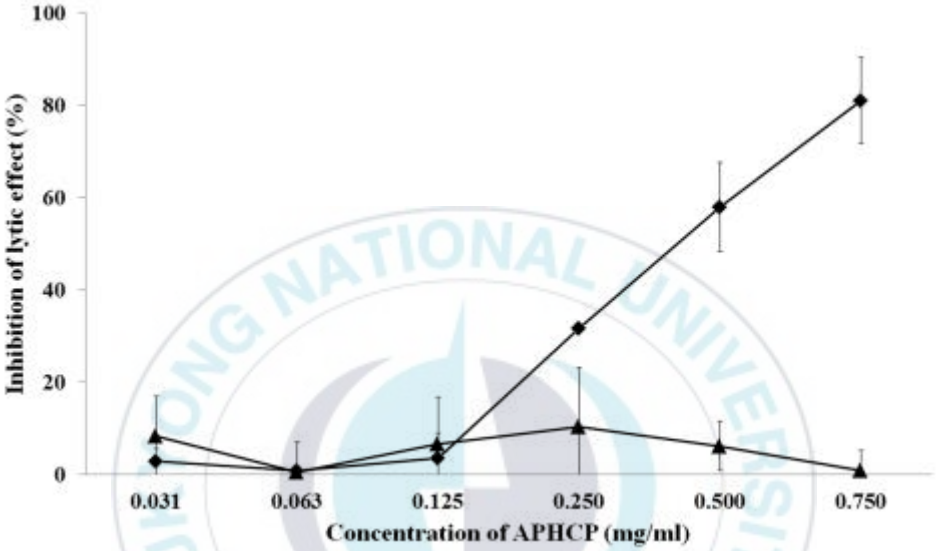

Figure 10. Inhibitory lytic effect of APPCP on HIV-1_{IIIB} infected MT-4 cells. MT-4 cells that treated with APPCP(▲) were incubated with HIV-1_{IIIB}. 84 h later, MTT was added and incubated for 4 h additionally to form formazan. Cell viability was detected by absorbance of dissolved formazan. Peptide-untreated and virus-uninfected MT-4 cells were used as control. (APHCP, ◆)

3.8. Effect of APHCP on HIV-2_{ROD} infection

In addition, we examined whether the inhibitory effect of APHCP is specific against HIV-1 infection, since AIDS is caused by HIV-1 as well as HIV-2. As shown in Fig. 11, APHCP did not show inhibitory effect on lysis of HIV-2_{ROD}-infected MT-4 cells. This result suggest that the anti-HIV activity of APHCP is specific against HIV-1 but not against HIV-2.

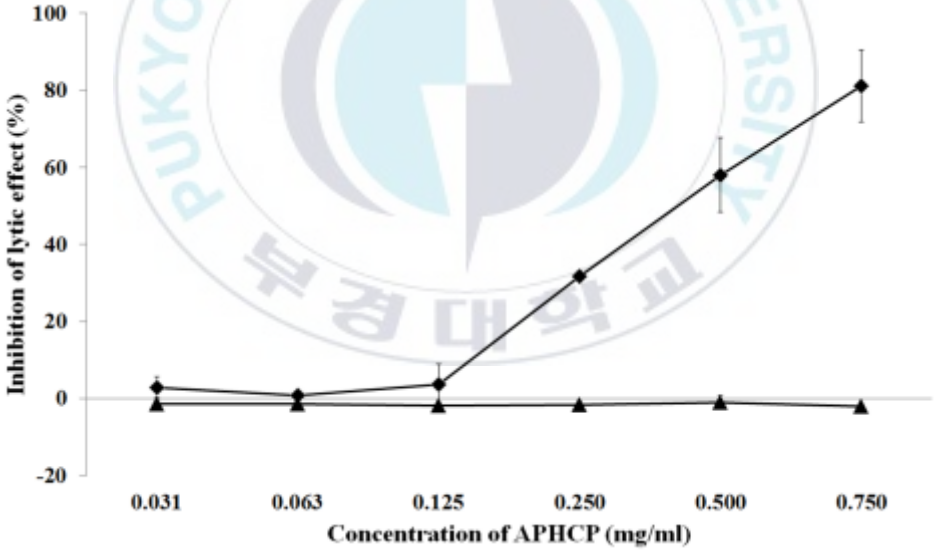

Figure 11. Effect of APHCP and APPCP on lysis of HIV-2_{ROD}- infected MT-4 cells. MT-4 cells were infected with HIV-1_{IIIB} or HIV-2_{ROD} in the presence or absence of various concentration APHCP(◆) or APPCP(▲). Cell lytic effect was measured by MTT assay.

4. Discussion

Currently, there is no cure and no preventive vaccine for HIV/AIDS. A major anti-HIV therapy employs the combinational use of at least three antiretroviral drugs to maximally suppress the HIV virus and stop the progression of HIV disease. The combinational antiretroviral therapy has dramatically improved treatment, but it has to be taken for a lifetime, has major side effects and causes viral resistance. Therefore, the identification of new classes of antiretroviral drugs with unique mechanisms of action remains an important therapeutic objective.

In this study, we suggest the potential of antioxidant marine peptide APHCP for more effective treatment and prevention of HIV. HIV infection has been believed to generate oxidative stress through production of reactive oxygen species (ROS). In patients infected with HIV-1, the levels of intracellular antioxidants such as GSH, cysteine, vitamin C, and SOD are decreased (Malvy et al., 1994; Fuchs et al., 1995; Peterhans 1997). The decrease in antioxidant levels results in the inhibition of intracellular virus replication (Sprietsma, 1997). Recent work has shown that HIV-1 can induces ROS production in astrocytes and microglia (Carroll-Anzinger et al., 2007; Reddy et al., 2012) and viral gp120 directly induces apoptosis in neurons (Ronaldson and Bendayan, 2008). The ROS-mediated blood-brain barrier damage in the HIV-1 infection has been also found to cause a loss of cell thigh junction proteins and lipid per oxidation (Reddy et al., 2012; Silverstein et al., 2011). Therefore, immune cells seem to require more antioxidants to maintain their function and integrity. Antioxidants may have a significant role in the treatment of HIV/AIDS.

APHCP, a peptide derived from gelatin hydrolysates of *Alaska pollack* skin has been

previously reported to have potent antioxidant activity on peroxidation of linoleic acid (Kim et al., 1984). In this study, we show that APHCP has a potent anti-HIV-1 activity. APHCP is not cytotoxic, even at exceedingly high concentration. Non cytotoxic APHCP exhibited inhibitory activity against HIV-1_{IIIB}-induced lytic effect, syncytia formation, viral p24 antigen production, and reverse transcriptase activity in MT4 cells. The anti-HIV activity of APHCP was found to be specific against HIV-1 but not against HIV-2. To know whether the anti-HIV-1 activity of APHCP is contributed by its antioxidant activity, we synthesized APPCP peptide that four hydroxyproline residues in APHCP are substituted by prolines. The APPCP failed to decrease HIV-1 infection to MT4 cells. Therefore, it is reasonable to suggest that a marine peptide, APHCP exhibits anti-HIV-1 activity by scavenging intracellular ROS increased during HIV-1 infection. APHCP should be a potential therapeutic agent for treating impaired immune system related to free radical oxidation.

However, additional study needs to address the critical role of APHCP on molecular association between HIV-1 infection and ROS generation, because all antioxidant agents seem not to exhibit anti-HIV activity as well as that APHCP does. In the present study, we examined anti-HIV activity of nine peptides reported to have antioxidant activity in vitro or in vivo and found that only APHCP exhibit anti-HIV activity. Therefore, more decisive mechanism by which APHCP blocks HIV infection should be addressed. APHCP is composed of ten amino acid residues of Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly. Several studies have reported that the repeat of tripeptide $(Gly\text{-}Pro\text{-}Hyp)_n$ is a main component of collagen degradation products and adopts triple-helical structure. The hypdroxyproline residues in the peptide contributes to stability and biological activity of collagen triple helix (Doig, 2008; Dai et al., 2008;

Raman et al., 2008; Squeglia et al., 2014). On the other hand, HIV infection has been known to cause collagen deposition (Estes, 2009; Diaz et al., 2011; Kusko et al., 2012). HIV infection induces an imbalance between MMPs and endogenous tissue inhibitors of MMPs (TIMPs), leading to remodeling of the extracellular matrix and HIV-associated pathology (Mastroianni and Liuzzi, 2007). Louboutin et al. (2011) have shown that HIV-1 gp120-induced ROS causes MMP upregulation. Additionally, the collagen triple peptide is able to interfere with binding of proMMP2 to fibril collagen and promote the release and activation of collagen-sequestered promatrixmetalloproteinase-2 (proMMP-2) which associated with the resolution of liver fibrosis via fibrotic matrix-sequestered gelatinases (Ruehl et al., 2011; Freise et al., 2012). Taken together, HIV infection-induced ROS generation promotes release and activation of collagen-sequestered proMMP2, resulting in accelerated collagen resolution, tissue damage, and collapse of immune system. On the other hand, the hydroxyproline-containing triple collagen peptide such as APHCP seems to prevent binding of proMMP2 to native collagen and release of proMMP2 bound to fibrillar collagen, resulting in reduced MMP2 activation, collagen stabilization, and immune cell homeostasis consistent with anti-HIV activation. Therefore, the functional role as the collagen triple peptide as well as the antioxidant activity might be important for the anti-HIV activity of APHCP in MT4 cells. Further studying the effect of APHCP on MMP2 activation and collagen remodeling may provide a clear insight in the possible mechanism that underline the anti-HIV activity of APHCP. The relevant findings would provide a basis for further investigation into the use of a marine peptide, APHCP as a potential therapeutic agent for HIV/AIDS treatment.

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