Nutraceuticals & Food
Vol. 8, p. 154~157 (2003)

Nutraceuticals and Food

Isolation of HIV-1 Protease Inhibiting Peptide from Thermolysin Hydrolysate of Manila Clam Proteins

Tae-Gee Lee† and Dong-Min Yeum*

Department of Marine Food Industry, Namdo Provincial College of Jeonnam, Jangheung 529-851, Korea
**Department of Food Processing & Baking, Yangsan College, Yangsan 626-740, Korea*

Abstract

A peptide inhibiting HIV-1 protease was isolated from the hydrolysate of manila clam (*Ruditapes philippinarum*) proteins digested with thermolysin. The peptide was purified by using membrane filtration, gel permeation chromatography, ion exchange chromatography, and reverse phase HPLC. The amino acid sequence of the peptide was determined to be Ile-Tyr-Glu-Gly. This tetrapeptide sequence exists in some proteins of *Physarum polycephalum* and *Mycobacterium smegmatis*. Chemically synthesized Ile-Tyr-Glu-Gly showed the IC_{50} value of 22.3 μM.

Key words: HIV-1 protease, manila clam protein, thermolysin hydrolysate

INTRODUCTION

The human immunodeficiency virus type-1 (HIV-1), the causative agent of acquired immunodeficiency syndrome (AIDS), belongs to the retrovirus family. The HIV-1 protease is a virally encoded protease that serves to cleave the *gag* and *gag-pol* polyprotein precusor into mature and functional proteins. This specific proteolysis occurs late in the viral life cycle and is essential for the maturation of the infectious virus (1). Therefore, HIV-1 protease inhibitors have been extensively investigated as antiviral agents for the control of HIV-1 infection (2-4). Analysis of the sequences of retroviral proteases led to the suggestion that these enzymes were members of the aspartic protease family, on the basis of the observed conservation of a characteristic Asp-Thr-Gly active site sequence (5-7). For this reason, HIV-1 protease was found to be inhibited *in vitro* by pepstatin A and acetyl-pepstatin (7-9). There are reports of HIV-1 protease inhibitors from natural sources such as α-microbial alkaline protease inhibitor (10), lignin-like substance derived from an edible mushroom, *Fuscoporia obliqua* (11,12), and native Korean plants (13). Saquinavir, ritonavir, indinavir, nelfinavir and amprenavir have been approved by the FDA and are being used in AIDS therapy in combination with reverse transcriptase inhibitors. However, the ability of the virus to generate resistant mutants (14,15) suggests that there is an ongoing need for new, structurally diverse HIV-1 protease inhibitors.

Thus, we searched for HIV-1 protease-inhibiting substance from manila clam extracts, and observed a HIV-1 protease-inhibiting activity in the thermolysin hydrolysate of manila clam proteins. This report describes the isolation and identification of the HIV-1 protease inhibitory peptide from the hydrolysate.

MATERIALS AND METHODS

Materials

Recombinant HIV-1 protease, and HIV protease substrate III (His-Lys-Ala-Arg-Val-Leu-*p*-nitro-Phe-Glu-Ala-Nle-Ser-NH_2) were purchased from Bachem Feinchemikalien AG (Switzerland). Ile-Tyr-Glu-Gly was synthesized at the Korea Basic Science Institute (Seoul, Korea). Manila clams were harvested from the southwestern coast of Korea.

In vitro assay for the HIV-1 protease inhibitory activity

An assay mixture containing 100 μL of buffer (100 mM sodium acetate, pH 4.9, 200 mM NaCl, 5 mM dithiothreitol, and 10% glycerol), 10 μL of a prepared sample and 25 μL of a solution of recombinant HIV-1 protease (0.020 mg/mL) was preincubated for 5 min at 37°C, and then 10 μL of substrate solution (1 mg/mL in H_2O) was added. The tube containing the mixture was incubated for 15 min at 37°C and the reaction was stopped by the addition of 15 μL of 10% trifluoroacetic acid (TFA). Then, 20 μL of the solution was applied to a Zorbax 300SB C_8 column (4.6×150 mm, Agilent Technologies) for reverse-phase HPLC (HP 1100, Hewlett Packard Co., USA) and was elu-

†Corresponding author. E-mail: tglee@namdo.ac.kr
Phone: +82-61-860-8763, Fax: +82-61-860-8655

ted with a linear gradient of acetonitrile (0~63%) in 0.1% TFA at a flow rate of 1.0 mL/min. The *p*-nitro-Phe-Glu-Ala-Nle-Ser-NH_2 released was measured by monitoring the absorbance at 300 nm. The IC_{50} value was defined as the concentration of HIV-1 protease inhibitor required for 50% inhibition of HIV-1 protease activity.

Purification and analysis of HIV-1 protease inhibitors from a thermolysin hydrolysate of manila clam proteins

One hundred grams of the fresh raw manila clams was boiled for 10 min in 300 mL of distilled water, then minced and homogenized. Thirty mL of the homogenized sample was added to 50 mL of Tris-HCl buffer (100 mM, pH 8.2, containing 10 mM $CaCl_2$), and then 32 mg of thermolysin was added. After 4.5 hr of digestion at 37°C, the reaction was terminated by boiling for 10 min at 100°C. The precipitate was removed by filtration with Toyo filter paper (Toyo Roshi Co., Ltd.), and the filtrate was then ultrafiltered with PM-10 membrane (Amicon Co.). The crude peptides were applied to a Sephadex LH-20 column (26 ×900 mm, Pharmacia Fine Chemicals) and eluted with 30% methanol at a flow rate of 22 mL/hr. The active fraction was collected and concentrated, and then applied to a SP-Toyopearl 650S column (16×650 mm, Tosoh Co.) equilibrated with distilled water and was eluted with a linear gradient of NaCl concentration (0 to 1 M) at a flow rate of 30 mL/hr. The active fraction was collected and concentrated, and then applied to a SuperQ-Toyopearl 650S column (16×650 mm, Tosoh Co.) equilibrated with distilled water and was eluted with a linear gradient of NaCl concentration (0 to 1 M) at a flow rate of 30 mL/hr. The active fraction was purified on a Lichrosphere RP-18 column (4.6×250 mm, Hewlett Packard Co.), which was eluted with a linear gradient of methanol (0 to 63%) at a flow rate of 0.8 mL/min. The active peak from the Lichrosphere RP-18 column was further purified on a μBondasphere C_{18} column (3.9×150 mm, Waters Co.), which was eluted with a linear gradient of acetonitrile (0 to 63%) in 0.1% TFA at a flow rate of 1.0 mL/min. Each chromatography separation was monitored by the absorbance at 210 nm. The amino acid sequence of the purified peptide was analyzed with a protein sequencer (Procise 491, Applied Biosystems, USA).

RESULTS AND DISCUSSION

Fig. 1 shows the elution profile of the Sephadex LH-20 chromatogram of the hydrolysate filtered with PM-10 membrane. The fraction of the most active peak (No. 41) was collected and concentrated, and put on the SP-Toyopearl 650S column (Fig. 2). The active fraction (No. 16) was obtained at the void column. It was further fractionated by SuperQ-Toyopearl 650S chromatography (Fig. 3). The fraction (No. 75) with HIV-1 protease inhibitory activity was collected. Then it was further purified by HPLC on a Lichrosphere RP-18 column (Fig. 4). Although many peaks were observed by this chromatograph, only one peak showed inhibitory activity. The peak was col-

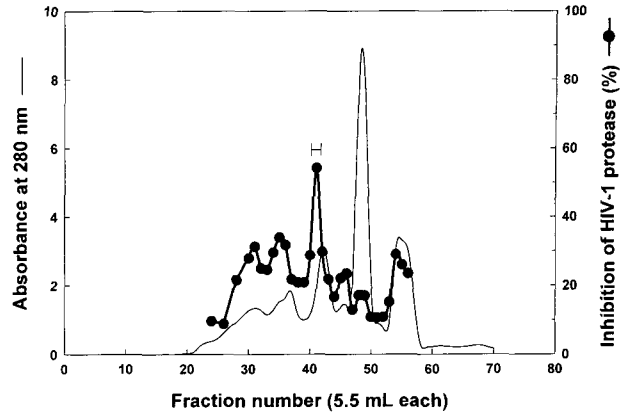

Fig. 1. A thermolysin hydrolysate of manila clam proteins filtered with PM-10 membrane was chromatographed on a Sephadex LH-20 column. The fraction marked with a horizontal line was collected.

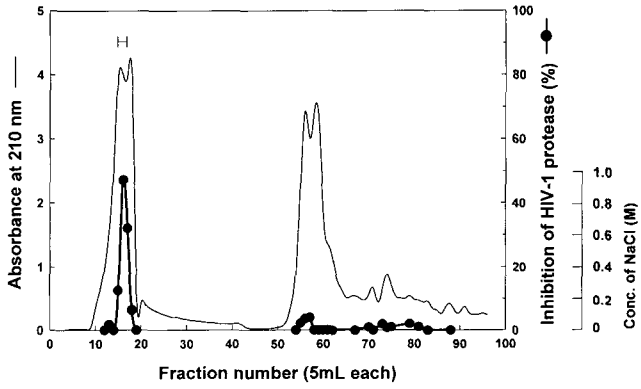

Fig. 2. The No. 41 fraction eluted from the Sephadex LH-20 column was chromatographed on a SP-Toyopearl 650S column. The No. 16 fraction was collected.

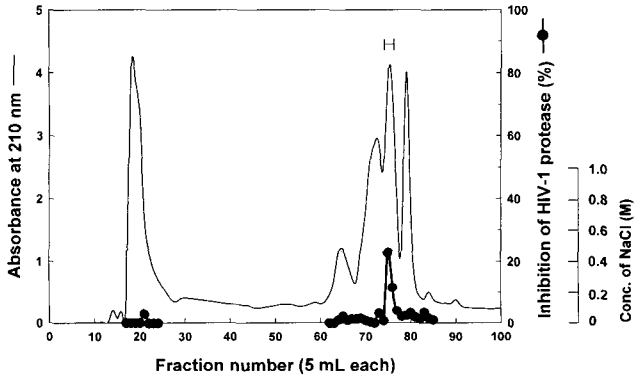

Fig. 3. The No. 16 fraction eluted from the SP-Toyopearl 650S column was chromatographed on a SuperQ-Toyopearl 650S column. The No. 75 fraction was collected.

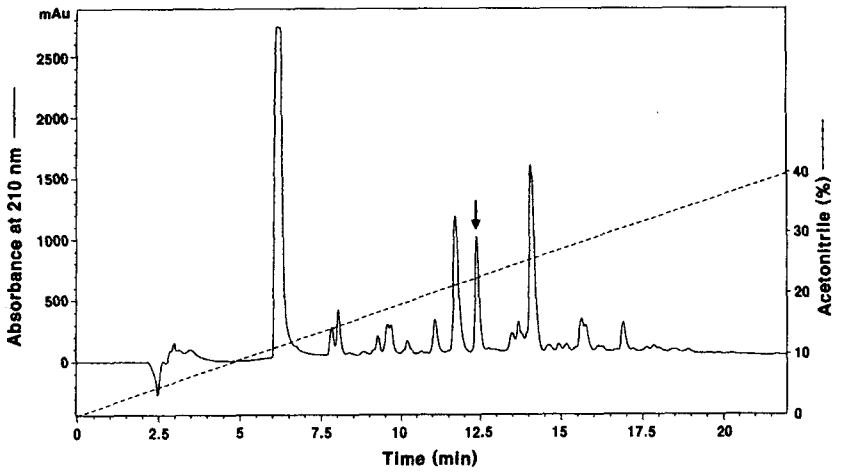

Fig. 4. Reverse-phase HPLC on a Lichrosphere RP-18 HPLC column of the active peak eluted the from the SuperQ-Toyopearl 650S column. The peak indicated with the arrow was collected.

lected, and purified finally by another HPLC on a μ Bondasphere C_{18} column (figure not shown). The inhibitory activity of the purified sample showed 37% with 17.2 μM for the HIV-1 protease.

The purified sample was analyzed for amino acid sequence by automated Edman procedure. The peptide was determined to be a tetrapeptide, Ile-Tyr-Glu-Gly. Ile-Tyr-Glu-Gly, which corresponds to the amino acid sequence 165~168 of *Physarum polycephalum* actin (16), and the amino acid sequence 25~28 of *Mycobacterium smegmatis* ferredoxin (17). A peptide with an identical amino acid sequence was synthesized and its HPLC profile was compared with that of purified one using a μBondasphere C_{18} column. Its retention time was similar to that of purified sample (results not shown).

The IC_{50} value of the synthetic Ile-Tyr-Glu-Gly was 22.3 μM. This peptide was approximately 10-fold more potent as a HIV-1 protease inhibitor than pepstatin A (IC_{50} = 250 μM) (7), a characteristic inhibitor of aspartic proteases. Since this peptide was not hydrolyzed by HIV-1 protease, we considered that the peptide is not substrate but an inhibitor. The HIV-1 protease inhibitory activity of the manila clam-derived peptide was rather low, compared with peptide-based synthetic inhibitors such as KNI-102 (IC_{50} = 89 nM) (2), KNI-272 (IC_{50} = 6.5 nM) (3), and saquinavir (IC_{50} = <0.4 nM) (4), but the peptide was more potent than other natural products.

Thus, the peptide purified from thermolysin hydrolysate of manila clam proteins was identified as a potent inhibitor of HIV-1 protease. Although the origin of this peptide is unclear, it is interesting that the sequence exists in some microbial proteins.

ACKNOWLEDGEMENTS

This work was supported by Korea Research Foundation Grant (KRF-2001-002-H00012).

REFERENCES

1. Kohl NE, Emini EA, Schleif WA, Davis LJ, Heimbach JC, Dixon RAF, Scolnick EM, Sigal IS. 1988. Active human immunodeficiency virus protease is required for viral infectivity. *Proc Natl Acad Sci USA* 85: 4686-4690.
2. Mimoto T, Imai J, Tanaka S, Hattori N, Kisanuki S, Akaji K, Kiso Y. 1991. KNI-102, a novel tripeptide HIV protease inhibitor containing allophenylnorstatine as a transition-state mimic. *Chem Pharm Bull* 39: 3088-3090.
3. Kageyama S, Mimoto T, Murakawa Y, Nomizu M, Ford H Jr, Shirasaka T, Gulnik S, Erickson J, Takada K, Hayashi H, Broder S, Kiso Y, Mitsuya H. 1993. *In vitro* anti-human immunodeficiency virus (HIV) activities of transition state mimetic HIV protease inhibitors containing allophenylnorstatine. *Antimicrob Agents Chemother* 37: 810-817.
4. Roberts NA, Martin JA, Kinchington D, Broadhurst AV, Craig JC, Duncan IB, Galpin SA, Handa BK, Kay J, Kröhn A, Lambert RW, Merrett JH, Mills JS, Parkes KEB, Redshaw S, Ritchie AJ, Taylor DL, Thomas GJ, Machin PJ. 1990. Rational design of peptide-based HIV proteinase inhibitors. *Science* 248: 358-361.
5. Toh H, Ono M, Saigo K, Miyata T. 1985. Retroviral protease-like sequence in the yeast transposon Ty*1*. *Nature* 315: 691.
6. Pearl LH, Taylor WR. 1987. A structural model for the retroviral proteases. *Nature* 329: 351-354.
7. Seelmeier S, Schmidt H, Turk V, von der Helm K. 1988. Human immunodeficiency virus has an aspartic-type protease that can be inhibited by pepstatin A. *Proc Natl Acad Sci USA* 85: 6612-6616.
8. Matayoshi ED, Wang GT, Krafft GA, Erickson J. 1990. Novel fluorogenic substrates for assaying retroviral proteases by resonance energy transfer. *Science* 247: 954-958.
9. Richards AD, Roberts R, Dunn BM, Graves MC, Kay J. 1989. Effective blocking of HIV-1 proteinase activity by characteristic inhibitors of aspartic proteinases. *FEBS Lett* 247: 113-117.
10. Stella S, Saddler G, Sarubbi E, Colombo L, Stefanelli S, Denaro M, Selva E. 1991. Isolation of α-MAPI from fermentation broths during a screening program for HIV-1

protease inhibitors. *J Antibiotics* 44: 1019-1022.
11. Ichimura T, Watanabe O, Maruyama S. 1998. Inhibition of HIV-1 protease by water-soluble lignin-like substance from an edible mushroom *Fuscoporia obliqua*. *Biosci Biotechnol Biochem* 62: 575-577.
12. Ichimura T, Otake T, Mori H, Maruyama S. 1999. HIV-1 protease inhibition and anti-HIV effect of natural and synthetic water-soluble lignin-like substances. *Biosci Biotechnol Biochem* 63: 2202-2204.
13. Hur JM, Park JG, Park JC, Hyun KH, Lee KY, Miyashiro H, Hattori M. 2002. Inhibition effects of ninety nine Korean plants on human immunodeficiency virus type 1 protease activity. *Nutraceuticals & Food* 7: 123-127.
14. Jacobsen H, Yasargil K, Winslow DL, Craig JC, Krohn A, Duncan IB, Mous J. 1995. Characterization of human immunodeficiency virus type 1 mutants with decreased sensitivity to proteinase inhibitor Ro 31-8959. *Virology* 206: 527-534.
15. Condra JH, Schlelf WA, Blahy OM, Gabryelski LJ, Graham DJ, Quintero JC, Rhodes A, Robbins HL, Roth E, Shivaprakash M, Titus D, Yang T, Teppler H, Squires KE, Deutsch PJ, Emini EA. 1995. In vivo emergence of HIV-1 variants resistant to multiple protease inhibitors. *Nature* 374: 569-571.
16. Vandekerckhove J, Weber K. 1978. The amino acid sequence of *Physarum* actin. *Nature* 276: 720-721.
17. Hase T, Wakabayashi S, Matsubara H, Imai T, Matsumoto T, Tobari J. 1979. *Mycobacterium smegmatis* ferredoxin. A unique distribution of cysteine residues constructing iron-sulfur clusters. *FEBS Lett* 103: 224-228.

(Received March 24, 2003; Accepted May 26, 2003)