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Notes

Peptide Inhibitor for Human Immunodeficiency Virus Type 1 (HIV-1) Protease from a Thermolysin Hydrolysate of Oyster Proteins

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A peptide that inhibits HIV-1 protease was isolated from a hydrolysate of oyster (*Crassostrea gigas*) proteins digested with thermolysin. The peptide was using membrane filtration, gel permeation chromatography, ion exchange chromatography, and reverse-phase high performance liquid chromatography. Amino acid sequence of the peptide was determined to be Val-Phe-Glu-Leu. Chemically synthesized Val-Phe-Glu-Leu showed an IC_{50} value of 106 μM.

Key words: HIV-1 protease, Oyster protein, Thermolysin hydrolysate, Oyster

Introduction

Human immunodeficiency virus type 1 (HIV-1), which causes AIDS (acquired immunodeficiency syndrome), is a member of the retrovirus family. HIV-1 protease is a virally encoded protease that cleaves the *gag* and *gag-pol* polyprotein precursor into mature, functional proteins. This specific proteolysis occurs late in the viral life cycle and is essential for the maturation of the infectious virus (Kohl et al., 1988). Therefore, HIV-1 protease inhibitors have been investigated extensively as antiviral agents for the control of HIV-1 infection (Roberts et al., 1990; Kageyama et al., 1993). These enzymes, which, based on the conservation of a characteristic Asp-Thr-Gly active site sequence, are considered members of the aspartic protease family (Toh et al., 1985; Pearl and Taylor, 1987), are inhibited *in vitro* by pepstatin A and acetyl-pepstatin (Seelmeier et al., 1988; Matayoshi et al., 1990; Richards et al., 1989). In addition, HIV-1 protease inhibitors are found in nature, such as α-microbial alkaline protease inhibitor (Stella et al., 1991), a lignin-like substance derived from an edible mushroom, *Fuscoporia oblique* (Ichimura et al., 1998; Ichimura et al., 1999), and native Korean plants (Hur et al., 2002). Saquinavir, ritonavir, indinavir, nelfinavir, and amprenavir are approved by the United States Food and Drug Administration (FDA) and are used in AIDS therapy in combination with reverse transcriptase inhibitors.

Some oysters are susceptible to infection by pathogenic bacteria and viruses. Hence, ingesting raw oysters can be dangerous to HIV-infected and other immunocompromised individuals (Chin et al., 1987; Ross et al., 1994). However, oysters should produce antiviral and antibacterial substances against infectious organisms. Interestingly, oysters exhibit free radical-scavenging activity (Yoshikawa et al., 1997) and potentiate interleukin-2 effects on immune cells (Achour et al., 1997). Thus, we searched for HIV-1 protease-inhibiting substances in oyster extracts, and observed HIV-1 protease inhibitory activity in a thermolysin hydrolysate of oyster proteins. The objective of this study was to isolate and identify the HIV-1 protease inhibitory peptide from oyster hydrolysate.

Materials and Methods

Recombinant HIV-1 protease and the 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). Val-Phe-Glu-Leu was synthesized at the Korea Basic Science Institute (Seoul, Korea). All other reagents were of high performance liquid chromatography (HPLC) grade or analytical grade. Oysters (*Crassostrea gigas*) were obtained from the coast of Jangheung province

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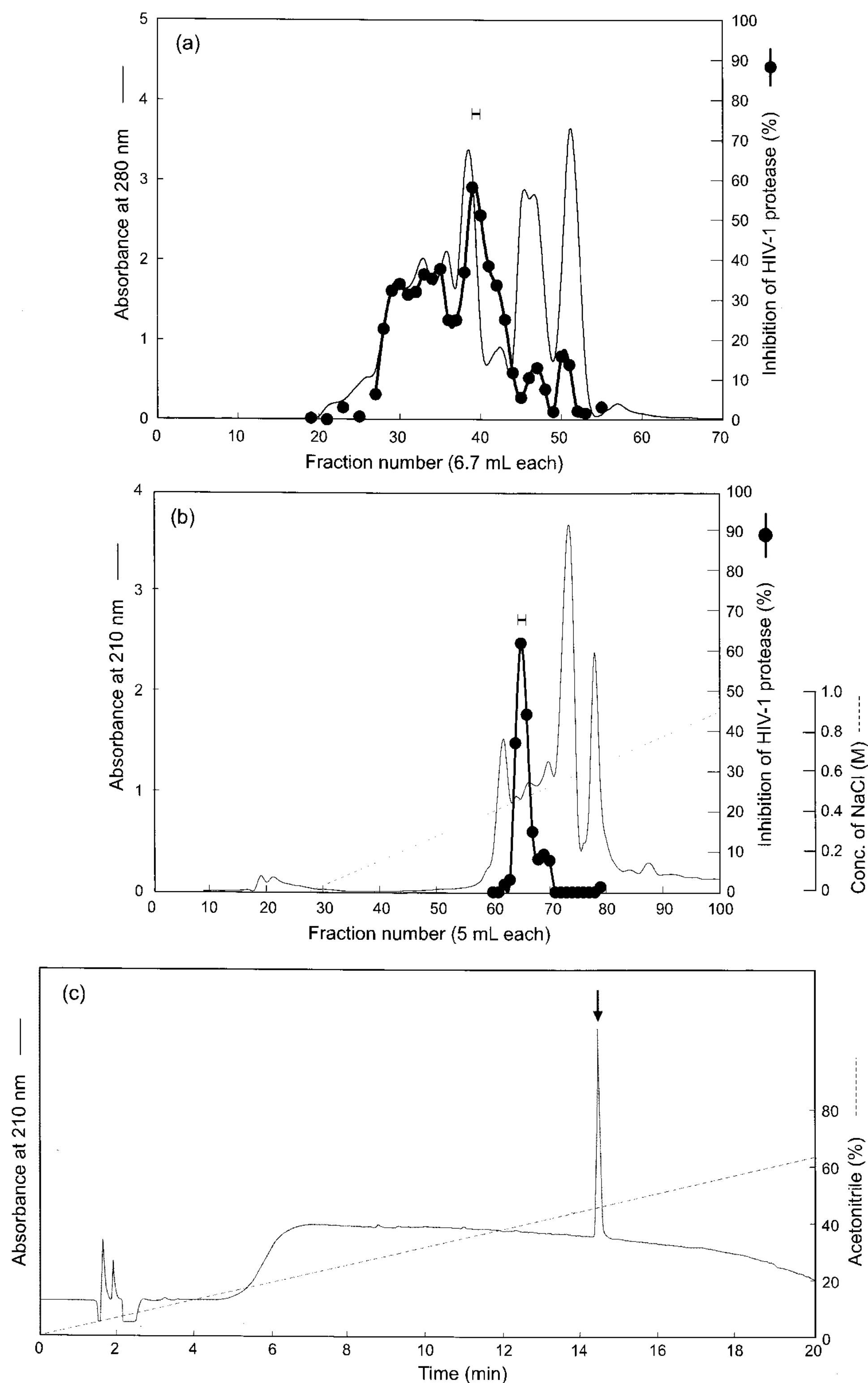

Fig. 1. Purification of Val-Phe-Glu-Leu from a thermolysin hydrolysate of oyster proteins. (a) A thermolysin hydrolysate of oyster proteins filtered with PM-10 membrane was chromatographed on a Sephadex LH-20 column. The fractions marked with a horizontal line were collected. (b) The No. 20 fraction eluted from the SP-Toyopearl 650S column was chromatographed on a SuperQ-Toyopearl 650S column. The No. 65 fraction was collected. (c) Reverse-phase HPLC on a Symmetry Shield RP-18 HPLC column of the active peak eluted from the μBondapak C_{18} HPLC column. The peak indicated with the arrow was collected.

located in southern Korea, in January 2003.

An assay mixture containing 100 μL 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 substrate solution (1 mg/mL in H_2O) was added. The mixture was incubated for 15 min at 37°C then the reaction was stopped by adding 15 μL of 10% trifluoroacetic acid (TFA). Next, 20 μL of the solution was applied to a μBondasphere C_8 column (3.9×150 mm; Waters Co.) for reverse-phase HPLC (HP 1100; Hewlett Packard Co., USA) and eluted with a linear gradient of acetonitrile (0 to 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.

One hundred grams of fresh raw oysters was boiled for 10 min in 300 mL distilled water, and then minced and homogenized. Then 30 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 32 mg thermolysin was added. After digestion for 4.5 h 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 then the filtrate was ultrafiltered using a 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 20 mL/h. The active fraction was collected and concentrated, and then applied to a SP-Toyopearl 650S column (16×650 mm; Tosoh Co., Ltd.) equilibrated with distilled water, and eluted with a linear gradient of NaCl (0 to 1 M) at a flow rate of 30 mL/h. The active fraction was collected and concentrated, and then applied to a SuperQ-Toyopearl 650S column (16×650 mm; Tosoh Co., Ltd.) equilibrated with distilled water, and eluted with a linear gradient of NaCl (0 to 1 M) at a flow rate of 30 mL/h. The active fraction was purified using a μBondapak C_{18} column (3.9×300 mm; Waters Inc.), eluting with a linear gradient of methanol (0 to 63%) at a flow rate of 0.8 mL/min. The active peak from the μBondapak C_{18} column was further purified on a Symmetry Shield RP-18 column (4.6×150 mm; Waters Inc.), eluting with a linear gradient of acetonitrile (0 to 63%) in 0.1% TFA at a flow rate of 1.0 mL/min. Each chromatography 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

The elution profile from the Sephadex LH-20 separation of the hydrolysate filtered with a PM-10 membrane is shown in Figure 1a. The fractions of the most active peak (No. 39-40) were collected, concentrated, and applied to the SP-Toyopearl 650S column. The active fraction (No. 20) was obtained at the void column. It was further fractionated by SuperQ-Toyopearl 650S chromatography (Fig. 1b). The fraction (No. 65) containing HIV-1 protease inhibitory activity was collected and further purified by HPLC using a μBondapak C_{18} column. Although many peaks were observed in this chromatograph, only one small peak showed inhibitory activity. The peak was collected, and purified by another HPLC step using a Symmetry Shield RP-18 column (Fig. 1c). The purified sample, at 103 μM, inhibited HIV-1 protease activity by 48%.

The purified sample was analyzed for its amino acid sequence using an automated Edman procedure. The peptide was determined to be a tetrapeptide, Val-Phe-Glu-Leu, which corresponds to amino acid sequence 63-66 of the encephalomyocarditis viral polyprotein (Palmenberg et al., 1984) and amino acid sequence 463-466 of the human influenza A virus nucleoprotein (Huddleston and Brownlee 1982).

The IC_{50} value of a synthetic Val-Phe-Glu-Leu was 106 μM (Table 1). This peptide is approximately twice as potent as the HIV-1 protease inhibitor than pepstatin A, a characteristic inhibitor of aspartic proteases. Pepstatin A and acetyl-pepstatin are competitive inhibitors of HIV-1 protease with *Ki* values of 17 and 20 nM at pH 4.7 (Matayoshi et al., 1990; Richards et al., 1989). The HIV-1 protease inhibitory activity of the oyster-derived peptide was rather weak compared to peptide-based synthetic inhibitors such as KNI-102 (IC_{50} 89 nM) and KNI-272 (IC_{50} 6.5 nM) (Kageyama et al., 1993), but more potent than other natural products.

Table 1. HIV-1 protease inhibitory activity of peptide

Peptide	IC_{50} (μM)
Val-Phe-Glu-Leu	106

In summary, the peptide purified from a thermolysin hydrolysate of oyster 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 other biological proteins.

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