Bioorganic & Medicinal Chemistry Letters 18 (2008) 3963–3967

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

A serine protease inhibitor from hemolymph of green mussel, *Perna viridis*

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ARTICLE INFO

Article history:
Received 8 April 2008
Revised 2 June 2008
Accepted 5 June 2008
Available online 10 June 2008

Keywords:
Perna viridis
Protease inhibitor
Natural product
Hemolymph

ABSTRACT

Bioactivity guided fractions of cell-free hemolymph of bacterially challenged marine mussel, *Perna viridis* led to the isolation of a novel quaternary alkaloid **1**, which was identified by its spectral data. The isolated molecule **1** has been found to be a potent serine protease inhibitor (SPI) showing IC_{50} and K_i values of 102.5 and 97.1–104.68 μM, respectively. The E_t/K_i value of SPI is 6.3, whereas E_t/K_m value is 1.04. The Van't Hoff analysis showed that the value of K_i decreases with increase in temperature, and the binding of the inhibitor is entropically driven.

Invertebrates possess a specific and innate immune mechanism, and are lacking in immune memory following the first encounter with a pathogen. Molluscs have humoral and cellular immunity, and the humoral system is constituted by lysosomal enzymes, agglutinins, lectins and antimicrobial peptides. Nevertheless, cellular immunity seems to perform the main role in shellfish immune processes.[1] Historically, one abundant source of novel therapeutic agents has been natural products.[2,3] Many of these have acted as pharmacophores or templates from which therapeutically useful agents have been designed. In fact, the first reported bioactive marine natural products, spongouridine and spongothymidine, served as templates for the development of cytosine arabinoside, an anticancer agent.[4] Halisulfate and suvanine inhibited the serine proteases thrombin and trypsin and are novel anticoagulants isolated from the marine animals.[5]

In vertebrates, serine protease inhibitors have been studied extensively and they are known to be involved in phagocytosis, coagulation, complement activation, fibrinolysis, blood pressure regulation, etc. In the last decade, it became obvious that in invertebrates, serine proteases and their inhibitors are also involved in parallel physiological processes, for example, the blood clotting cascade in *Limulus*[6] and the innate immune response.[7] Moreover, some of the protease inhibitors isolated from invertebrate sources are quite specific towards individual mammalian serine proteases.

Perna viridis, asian green mussel, is a large (>80 mm) bivalve, with a smooth, elongate shell. The native range of the Asian green mussel broadly encompasses the Asia-Pacific and Indo-Pacific regions.[8] Primarily, the green mussel has been utilized as a pollution control indicator.[9–11] However, it has not been chemically examined for its bioactive constituents. In this endeavor, we have undertaken systematic activity guided isolation of bioactive constituents from this mussel for protease inhibitors.

The mussels were collected from western coast of Goa, India and maintained under laboratory conditions in sea water in an aerated glass aquarium. The mussels were acclimatized for 15 days before the start of experiment. Mussels (80–100 mm in length) were used as a source of hemolymph. Humoral immune responses were elicited by injecting the combination of G^+ and G^- human pathogenic bacteria in 0.85% brine solution into the posterior adductor muscles. The bacteria used were G^+ (*S. aureus, S.* Group D) and G^- (*P. vulgaris*, *E. coli, P. aeruginosa, K. pneumonie, S. morganii*). The bacteria were grown separately in the LB (Luria–Bertani) broth till the OD 0.6 at 660 nm was reached. Grown culture (4 mL) was taken, and bacterial cells were pelleted by centrifugation at 12,000 rpm at 4 °C for 15 min. These pellets were washed twice with 0.85% NaCl (saline buffer). The pellets of different bacteria were mixed and dissolved in 1500 μl saline buffer. Animals were injected with 100 μl of mixed cocktail of bacteria into the posterior adductor muscles. Hemolymph collection was done by slightly opening the animals with the help of forceps so that adductor muscle does not break and then hemolymph collected from posterior adductor muscles. Hemolymph was collected into an Alsevier solution and immediately centrifuged at 1000 rpm at

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doi:10.1016/j.bmcl.2008.06.010

4 °C for 15 min and the supernatant collected was pooled and stored at −80 °C for further use.

Frozen cell-free immune hemolymph (500 mL) were thawed and subjected to ultrafilteration by using 3 kDa membrane. The filtrate below 3 kDa were lyophilized to dryness and subjected to organic solvent extraction. The lyophilized hemolymph (42 g) was exhaustively partitioned between petroleum ether (3× 300 mL), chloroform (3× 300 mL), ethyl acetate (3× 300 mL) and MeOH (3× 300 mL). Each step of fractionation was monitored by the in vitro serine protease assay. The MeOH fraction (3 g) was loaded on silica gel column and eluted with $CHCl_3$:MeOH (0→5%). Fractions eluting with 3% MeOH–$CHCl_3$ containing bioactive violet spots were pooled together and subjected to preparative TLC (0.25 mm, 20 × 20 cm, SiO_2 GF_{254}, Merck, India) and developed in a solvent system of butanol/acetic acid/water (4:4:2). The crude product so obtained was finally purified (10 mg) by RP-HPLC (C_{18} symmetry, MeCN (5→95%):H_2O R_t 10 min, detection at 214 and 280 nm).

Compound **1** was obtained as an amorphous solid, *m*/*z* 355.0279 ($[M]^+$), $C_{16}H_9O_8N_2$. The infrared (*IR*) spectrum of **1** suggested the presence of aromatic C–H stretching (2925, 2854 cm^{-1}), tertiary amine, C=N stretching (1687.48, 1666.80 cm^{-1}) and carbonyls (1687, 1666 cm^{-1}). The ^1H NMR spectrum of **1** showed the presence of five aromatic protons at δ 7.59 dd (2H, J = 8.5, 7.0 Hz), δ 7.69 dd (1H, J = 7.6, 7.2 Hz) and δ 7.79 d (2H, J = 7.6 Hz) and a D_2O exchangeable broad singlet at 9.15 ppm integrating for four protons corresponding to four carboxylic protons. The ^{13}C NMR spectrum of **1** showed 9 carbon signals at 116.0, 118.0, 128.5, 128.8, 129.5, 134.2, 158.0, 159.0 and 166.2 ppm. The carbon signals appearing at 116.0, 118.0, 128.8, 158.0, 159.0 and 166.3 ppm were found to be of quaternary nature by its ^{13}C DEPT. The proton signal appearing at δ 7.79 in the ^1H–^1H–COSY spectrum showed the connectivity with the proton signal at δ 7.59 and 7.69. The structure was further confirmed by its HMBC ^1H–^{13}C spectrum, and the connectivities have been shown in Figure 2. The multiplet at 7.59, 7.69 and 7.77 ppm showed the correlations with the carbon signals appearing at 128.5, 129.5 ppm and specially the proton signals at 7.77 ppm showed further correlation with 134.2 and a carbonyl frequency appeared at 166.2.

On the basis of ^{13}C NMR, DEPT, and 2D NMR and LC–MS spectra, the isolated compound has been identified as **1** (Fig. 1).

The kinetic parameters for the substrate hydrolysis were determined by measuring the initial rate of enzymatic activity. The inhibition constant K_i was determined by Dixon[12] method and also by the Lineweaver–Burk (LB) equation. The K_m value was also calculated from the double-reciprocal equation by fitting the data into the computer software ORIGIN 6.1. For the Lineweaver–Burk analysis, trypsin (612 μM) was incubated with inhibitor at 60 and 120 μM and assayed at increasing concentrations of BAPNA (*N*-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride)[13–16] (300–1250 μM) at 37 °C for 30 min. The reciprocals of substrate hydrolysis (1/v) for each inhibitor concentration were plotted against the reciprocals of the substrate concentrations, and the K_i was determined by fitting the resulting data. In Dixon's method, hydrolytic activity of trypsin (612 μM) was measured in the presence of 400 and 800 μM BAPNA, at concentrations of inhibitor ranging from 60 to 140 μM at 37 °C for 30 min. The reciprocals of substrate hydrolysis (1/v) were plotted against the inhibitor concentration and the K_i was determined by fitting the data using ORIGIN 6.1.

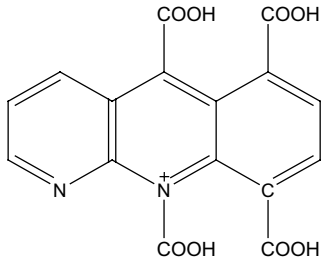

Figure 1. Structure of isolated bioactive compound **1**.

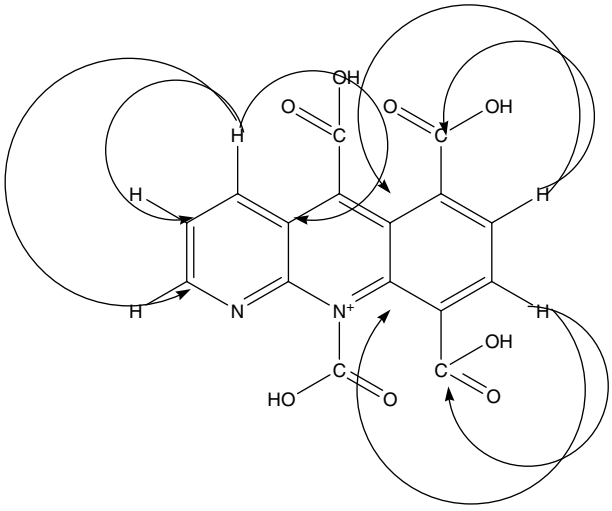

Figure 2. Complete HMBC correlations in compound **1**.

The inhibitor was found to inhibit trypsin with an IC_{50} value (50% inhibitory concentration) of 102.5 μM by fitting the data using ORIGIN 6.1 (Fig. 3). The inhibition of trypsin followed a hyperbolic pattern with increasing concentrations of the inhibitor. However, the secondary plot (the slope of inhibition graph versus inhibitor concentration) was not linear, suggesting that the application of Michaelis–Menten inhibition kinetics was not appropriate in this study. The inhibition constant K_i determined by the classical double reciprocal plot was 104.68 μM and by Dixon plot was 97.1 μM (Fig. 4), which is almost equal to the IC_{50} value of the inhibitor. The Line Weaver–Burk reciprocal plot (Fig. 5) showed that inhibitor was a competitive inhibitor of trypsin and the K_m value for the trypsin with BAPNA was 588 μM, and K_i for the inhibitor was 104.68 μM. For the kinetic analysis and rate constant determinations, the assays were carried out in triplicate, and the average value was considered throughout this work.

The inhibitor was found to be specific for trypsin and did not show any inhibition against chymotrypsin. It also did not show any activity against other classes of proteases. Goldstein[18] showed that the ratio [E_t (Total conc. of enzyme)/K_i] should be 0.01 or less for a Michaelis–Menten analysis to be valid at sub-saturating concentrations of inhibitor, if E_t/K_i exceeds 100 then virtually all of the added inhibitor molecules become bound to the enzyme and at intermediate E_t/K_i values, the total inhibitor is distributed between molecules free in solution and those complexed with the enzyme.[17] By a similar argument, the ratio E_t/K_m reveals the validity of the Michaelis–Menten treatment for rates obtained with substrate alone in the system.[17–19] The E_t/K_i value of SPI is 6.3 and for E_t/K_m is 1.04.

Free energy changes of trypsin inhibition against SPI (ΔG) were determined by

$$\Delta G = -RT \ln K_i \quad (1)$$

Temperature dependence of the inhibition constants was used to determine the thermodynamic parameters. Changes in enthalpy (ΔH) were determined from the Van't Hoff plots by using

$$\ln K_i = (\Delta H/RT) + \Delta S/R \quad (2)$$

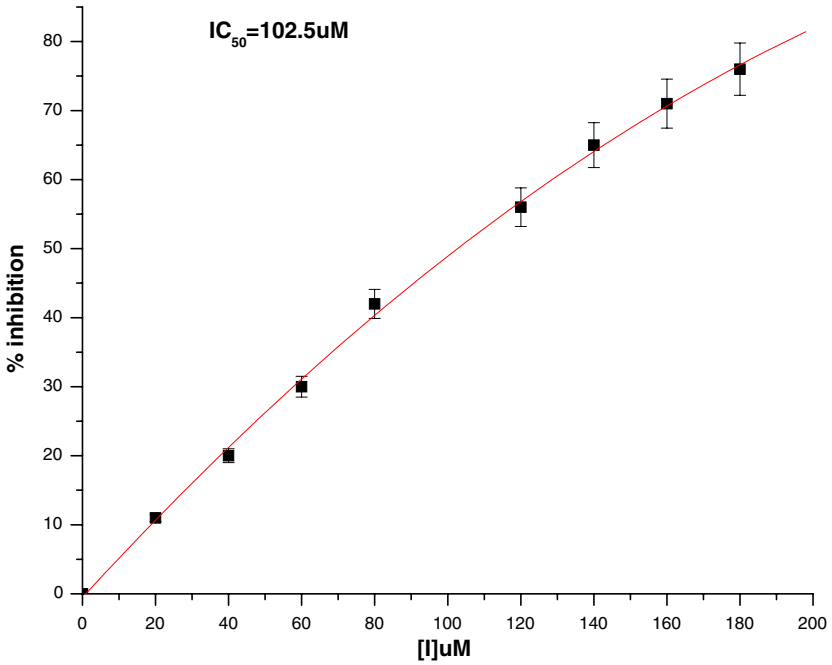

Figure 3. The sigmoidal curve indicates the best fit for the percentage inhibition data obtained. The IC_{50} value was calculated from the graph.

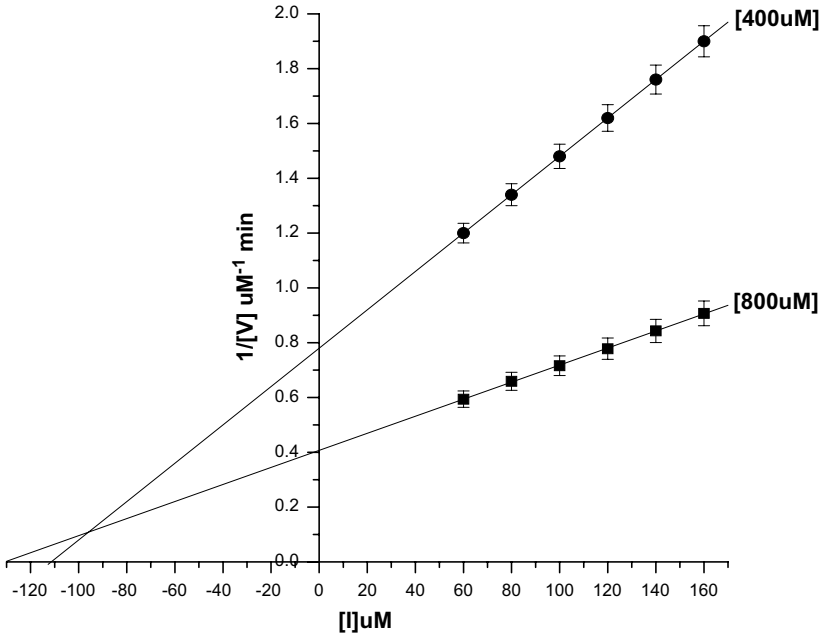

Figure 4. Enzymatic activity of the trypsin (612 μM) was estimated using the substrate BAPNA = 400 μM (•) and 800 μM (■) at different concentrations of SPI. Reciprocals of the reaction velocity were plotted versus the SPI concentration. The straight lines indicated the best fit of the data obtained. The inhibition constant K_i was calculated from the intersection point of the two plots.

where ΔH is enthalpy change, R is gas constant, ΔS is entropy change and T is the absolute temperature. The entropy change was obtained from

$$\Delta G = \Delta H - T\Delta S \quad (3)$$

As can be seen from Van't Hoff plot (Fig. 6), the inhibition of trypsin increases with increase in temperature and binding is endothermic. The free energy (ΔG) of binding of the inhibitor is negative (Table 1), and therefore the reaction is spontaneous. However, if the enthalpy ($-\Delta H$) and entropy (ΔS) is taken into consideration, the major contribution to free energy ($-\Delta G$) comes from entropy (ΔS or $-T\Delta S$), and therefore the binding of the inhibitor is entropically driven.

In conclusion, we have isolated a new quaternary alkaloid **1** from the green mussel, *P. viridis*, which was found to be a SPI having an IC_{50} value of 102.5 μM and K_i 97.1–104.68 μM.

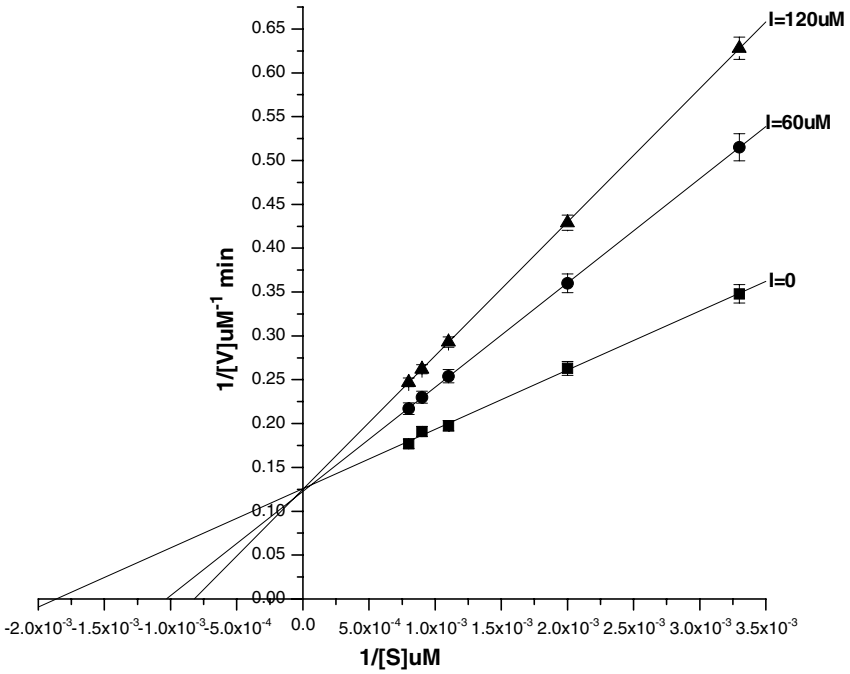

Figure 5. Trypsin (612 μM) was incubated without (■) or with SPI at 60 μM (•) and 120 μM (▲) concentrations and assayed at increasing concentrations of the substrate. The reciprocals of the rate of substrate hydrolysis for each inhibitor concentration were plotted against the reciprocals of the substrate concentrations. K_i was determined from the formula as per the competitive type of inhibition.

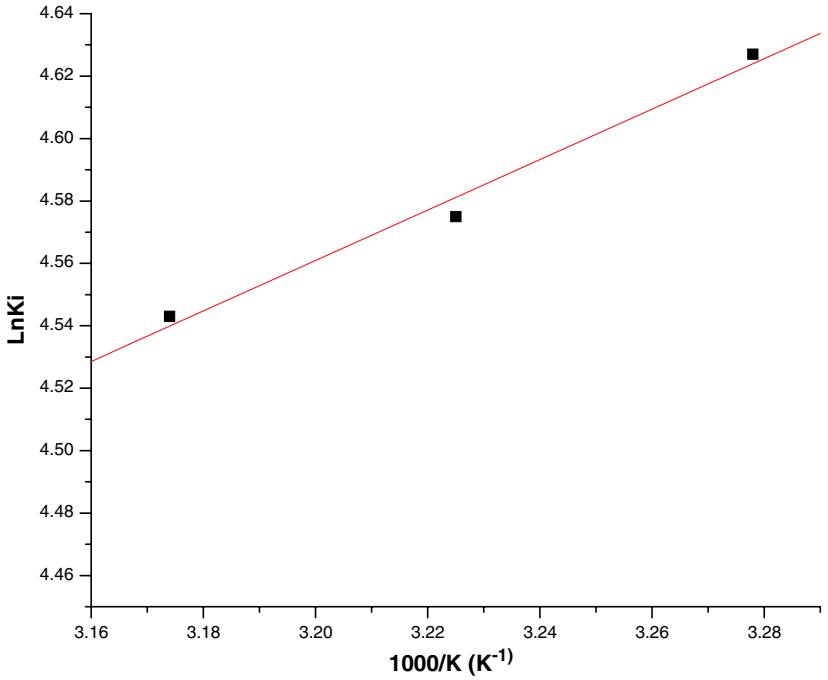

Figure 6. Van't Hoff plot of the effect of temperature on the inhibition constant of trypsin by SPI.

Table 1
Inhibition constant and thermodynamic parameter for inhibition of trypsin by SPI at different temperatures

Temperature (°C)	K_i (μM)	ΔG (kJ mol^{-1})	ΔH (kJ mol^{-1})	ΔS (J mol K^{-1})
32	102.3(±5.1)	−11.7(±0.5)	−2.0(±0.08)	31.9(±1.4)
37	97.1(±4.3)	−11.7(±0.4)	−2.0(±0.09)	31.1(±1.2)
42	94.0(±4.4)	−11.8(±0.5)	−2.1(±0.08)	31.0(±1.4)

(±) Standard deviation; $n = 3$.

Acknowledgements

M.S.K. thanks the Department of Biotechnology (DBT) and the Government of India, for their financial assistance.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.06.010.

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