Isolation of Nicotianamine as a Gelatinase Inhibitor

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Recent studies on matrix metalloproteinases (MMPs) have revealed that they play an important role in pathogenesis such as in angiogenesis, rheumatoid arthritis, and tumor invasion[1]. Among these enzymes, gelatinases are characteristic because of their ability to degrade type IV collagen, a major component of basement membrane, and have been focused on as pharmacological targets since the enzyme activities are elevated in these phathogenic states. Therefore, specific inhibitors of gelatinases would have clinical potential and thus we have been continuing to screen for low molecular weight inhibitors of gelatinases from microbial origin[2,3]. Nicotianamine, formerly isolated as a metal chelator from tobacco leaves[4], was found during this screening in the culture broth of *Streptomyces* sp. SANK 62595. In this paper, fermentation and isolation of nicotianamine and characteristics of enzyme inhibition by nicotianamine and a related compound, mugineic acid, are reported.

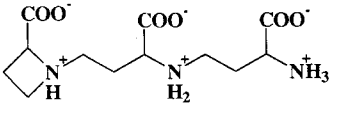
Fermentation of nicotianamine was carried out by the same method used for that of matlystatins[2], gelatinase inhibitors. Isolation of the active compound was achieved by monitoring the inhibitory activity against gelatinase B. The culture fluid (28 liters), separated from mycelium by filtration, was passed through a column of activated carbon (3 liters, 20 i.d. × 50 cm). The flow-through fraction was placed onto a column of Dowex SBR-P (10 liters, 23 i.d. × 140 cm, HCO_3^-). The column was washed with 100 mM NH_4HCO_3 (30 liters) and the active principle was eluted with 300 mM NH_4HCO_3 (30 liters). To remove the bulk of sodium bicarbonate present in the active fraction, pellets of Dowex 50WX4 (H^+) were added to trap ammonium ions and eventually to evolve carbon dioxide. The addition of the ion exchange resin was continued until carbon dioxide no longer evolved. The resulting solution was concentrated and lyophilized to give 21.0 g of a brownish powder. When one third of the powder (7.2 g) was dissolved in 720 ml of water, the ionic strength of the solution was still too high to perform the following isolation procedures. Thus, the residual salt was removed using mixed bed type ion exchange resin. The solution was passed through a column of Muromac MBX8 (120 ml, 1.5 i.d. × 75 cm, H^+ and OH^-, Muromachi Kagaku Kogyo Kaisha, Ltd.,) and the column was washed with 360 ml of water. The column size was adjusted to slightly less than the exchange capacity for the loaded sample. This treatment reduced the conductivity of the aqueous solution from 9.2 mS/cm to 1.3 mS/cm which is low enough for completion of the following treatment, and the active principle was recovered in a flow-through fraction without loss of the activity. The resulting solution was then applied onto a column of Dowex 1 × 2 (300 ml, 2.5 i.d. × 75 cm, HCO_3^-) equilibrated with 20 mM NH_4HCO_3. After the column was washed with 75 ml of 20 mM NH_4HCO_3, the active principle was eluted with a linear gradient of NH_4HCO_3 (20 mM ~ 300 mM, 1000 ml each). Active fractions were combined and lyophilized to give 38.2 mg of a white powder. This material was dissolved in water (5 ml) and chromatographed on a column of DEAE Sephadex A25 (35 ml, 1.5 i.d. × 20 cm, HCO_3^-) equilibrated with 20 mM NH_4HCO_3. After the column was washed with 20 mM NH_4HCO_3 (10 ml), the active principle was eluted with a linear gradient of NH_4HCO_3 (20 ~ 250 mM, 200 ml each). Active fractions were combined and lyophilized to give 7.8mg of a powder. The material was finally purified by preparative reversed phase HPLC column chromatography (Polyhydroxyethyl Aspartamide column, Poly LC Inc., Md., U.S.A., 4.6 i.d. × 200 mm, flow rate 1 ml/minute, solvent 60%MeCN-10 mM NH_4HCOOH). The active compound was eluted at 9 minutes and the active effluent was concentrated and lyophilized to yield approximately 2 mg of pure sample.

The molecular formula of the active compound was determined to be $C_{12}H_{21}N_3O_6$ by HRFAB-MS. A detailed analyses of ^1H and ^{13}C NMR spectral data revealed that this compound was identical to nicotianamine, formerly isolated from leaves of tobacco plants as a metal chelator (Fig. 1).

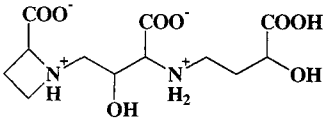
The characteristics of enzyme inhibition of nicotianamine was evaluated along with mugineic acid[5] (Fig. 1), a related compound isolated from the roots of barley. This compound also showed metal chelation activity.

The maximal inhibition of gelatinase B achieved by nicotianamine and mugineic acid was approximately 65% with apparent IC_{50} values of 0.23 μM and 1.0 μM,

Fig. 1. The structures of nicotianamine and mugineic acid.

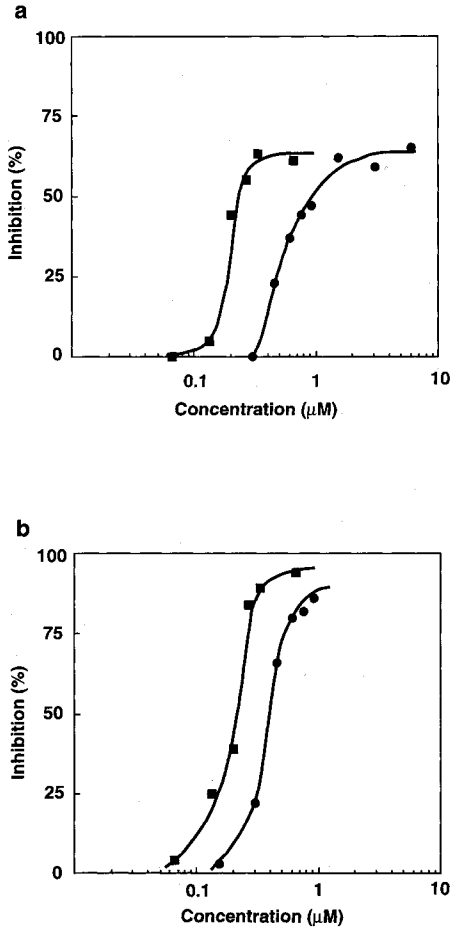


Nicotianamine



Mugineic acid

Fig. 2. Inhibition curves of gelatinase B (a) and A (b) by nicotianamine (closed square) or mugineic acid (closed circle).



respectively (Fig. 2a). In the case of gelatinase A, almost complete inhibition was achieved by nicotianamine and mugineic acid with IC_{50} values of 0.20 μM and 0.41 μM, respectively (Fig. 2b). On the other hand, they did not inhibit thermolysin and aminopeptidase M, other zinc metalloproteinases, up to 60 mM (data not shown).

To examine the reversibility of the inhibition by nicotianamine, gelatinase B was preincubated with varying concentrations of nicotianamine at 37°C for 1 hour, and the concentrations of nicotianamine were reduced to one fifth in the enzyme assay. As shown in Table 1, the inhibition by nicotianamine was reversible, as it did not depend on the nicotianamine concentration at preincubation but on that at enzyme assay, although the inhibition rate of nicotianamine was increased approximately 20% by preincubation with the enzyme at all nicotianamine concentrations. Since nicotianamine and mugineic acid were metal chelators[4,5], zinc ion was

Table 1. Reversible inhibition of MMP-9 by nicotianamine.

Concentration of nicotianamine (μM)		Relative activity (%)
At preincubation	At enzyme assay	
0	0	100
0	0.033	100
0	0.066	97
0	0.165	45
0	0.33	31
0	0.66	29
0.165	0.033	77
0.330	0.066	76
0.825	0.165	21
1.65	0.33	11
3.30	0.66	7

added at enzyme assay with a fixed concentration of nicotianamine. The inhibition of gelatinase B by nicotianamine was arrested by the addition of zinc ion dose dependently, and zinc ion equivalent to nicotianamine in the molar ratio completely overcame the inhibitory activity of nicotianamine. These results indicate that nicotianamine inhibits gelatinases with its chelating activity on the zinc ion existing in the catalytic center of gelatinases. It should be noted, however, that both compounds inhibit neither thermolysin nor aminopeptidase M. Nicotianamine and mugineic acid will be, therefore, useful probes for the structural study of gelatinases.

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