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# Inhibition of Various Proteases by MAPI and Inactivation of MAPI by Trypsin

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**Abstract** MAPI (microbial alkaline protease inhibitor) was isolated from culture broth of *Streptomyces chromofuscus* SMF28. The $K_i$ values of MAPI for the representative serine proteases such as chymotrypsin and proteinase K were 0.28 and 0.63 μM, respectively, and for the cysteine proteases cathepsin B and papain were 0.66 and 0.28 μM, respectively. These data indicate that MAPI is not a potent selective inhibitor of serine or cysteine proteases. Progress curves for the inhibition of three proteases by MAPI exhibited characteristic patterns: MAPI exhibited slow-binding inhibition of cathepsin B. It was rapidly associated with chymotrypsin before the addition of substrate and then reactivation of MAPI-inhibited enzyme was investigated in the presence of substrate. On the other hand, MAPI-proteinase K interaction was typical for those classical inhibitors. When MAPI was incubated with trypsin, there was an extensive reduction in the inhibitory activities of MAPI corresponding to 66.5% inactivation of MAPI, indicating that trypsin-like protease may play a role in the decrease of the inhibitory activity during cultivation.

**Key words:** MAPI, *Streptomyces chromofuscus*, slow-binding inhibition, inactivating enzyme, trypsin-like protease

A peptidic protease inhibitor, having a potent inhibitory action against subtilisin, was first isolated from culture filtrate of *Streptomyces nigrescens* WT-27 [13, 14]. This compound also inhibited various microbial alkaline proteases and is known as the microbial alkaline protease inhibitor (MAPI). MAPI was identified as a mixture of three compounds (designated as α-, β-, and γ-MAPI), which showed the same inhibitory spectra but different inhibitory potentials [24]. α- and β-MAPI were identified to be optical isomers with different configurations in the C-terminal phenylalaninal moiety [17, 25].

It has been known that MAPI had inhibitory activities against α-chymotrypsin, papain, ficin, bromelain, and cathepsin B, but not against trypsin, elastase, kallikrein, thermolysin, and pepsin [24]. Among the peptide aldehyde type protease inhibitors of microbial origin, MAPI had similar inhibitory specificities toward the chymotrypsin inhibitor, chymostatin, which inhibited α-chymotrypsin and papain [21], but not microbial alkaline proteases. Recently, it was reported that α-MAPI isolated from *Streptomyces* sp. GE16457 inhibited HIV-1 aspartic protease [19].

α-MAPI has been used to investigate the roles of intracellular chymotrypsin-like protease and cysteine proteases in the growth of rat embryo [7] and in the ovulation of hamster ovary [6]. It has been known that α-MAPI selectively inhibited sporulation of *Bacillus subtilis* by repressing proteolytic activity of the membrane bound serine protease through the complex formation [16].

As a specific inhibitor for serine and cysteine proteases, the inhibitory activity of MAPI has not yet been analyzed by kinetic analysis, therefore the present paper reports for the first time the kinetic measurements of the inhibitory activity of MAPI against chymotrypsin, proteinase K, cathepsin B, and papain as representative serine and cysteine proteases. Furthermore, as a preliminary investigation on the inhibition mechanism, the interaction of MAPI with proteases is described in the present study.

Little is known about the precise function of MAPI in the producing strains, but a factor inactivating MAPI was proposed in this study and this might be useful in elucidating the function of MAPI.

## MATERIALS AND METHODS

### Microorganism

*Streptomyces chromofuscus* SMF28 isolated from soil was used to produce MAPI [10]. The strain was transferred to

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slopes of stock culture medium each month and stored at 4°C.

### Media and Culture Conditions

Stock culture medium consisted of 1% glucose, 2% soluble starch, 0.5% yeast extract, 0.5% NZ-amine Type A (YTT), 0.1% $CaCO_3$, and 1.8% agar. Seed culture medium consisted of 3% glucose, 2% yeast extract, and 0.1% $CaCO_3$. The main culture medium consisted of 3% glucose, 2% casitone, 0.01% $KH_2PO_4$, 0.034% $K_2HPO_4$, 0.03% NaCl, 0.03% $MgSO_4 \cdot 7H_2O$, 0.001% $ZnCl_2$, 0.003% $MnCl_2 \cdot 4H_2O$, 0.001% $CaCl_2 \cdot 2H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, and 0.001% $CuSO_4 \cdot 7H_2O$. A loopful of spores from the slopes was inoculated into 100 ml of seed culture medium in a 500-ml baffled flask, and cultured at 30°C for 48 h in a rotary shaking incubator at 150 rpm. The seed culture at 5% (v/v) level was used to inoculate 3 l of the main culture medium in a 5-l jar fermentor (Korea Fermentor Co., Korea). Temperature was maintained at 30°C and culture pH was maintained at 7.0 by the addition of either 1 N HCl or 1 N NaOH automatically. Agitation and aeration were fixed at 300 rpm and 0.5 vvm, respectively.

### Instrumental Analysis

The inhibition kinetics were carried out on a UV-160 (Shimadzu, Japan) UV/VIS spectrophotometer. Mass spectral data was taken on a JMS AX505WA (Jeol, Japan) spectrometer. $^1$H-NMR spectral data was recorded with a AMX500 (Bruker, Germany) FT-NMR spectroscope using $CD_3OD$ as the solvent at ambient temperature.

### Analysis of Growth and Determination of Glucose and Ammonium Ion Concentrations

Mycelia in submerged culture were collected through vacuum filtration (GF/C filter paper, Whatman International Ltd., U.K.), dried at 80°C for 24 h, and the dry weight then was measured. The concentration of glucose was measured by the dinitrosalicylic acid method [12] and that of ammonium ion was determined by a specific ion analyzer (Model EA940, Orion Research, U.S.A.).

### Enzyme Assays

During cultivation and purification of the inhibitor, the amount of the inhibitor was estimated from the inhibitory activity against cathepsin B. Cathepsin B activity was assayed using *N*-α-benzoyl-DL-arginine-2-naphthylamide as substrate by the method of Barrett [3, 4]. Half a unit of cathepsin B was used in the following reaction mixtures (one unit of cathepsin B activity was defined as the amount to liberate 1 nmole of 2-naphthylamine from substrate per min at 37°C). A 10 µl volume of the inhibitor was preincubated with 100 µl of the enzyme in 0.1 M sodium phosphate buffer (pH 6.0) containing 1.33 mM EDTA/2 mM cysteine for 10 min, and then the reactions were started by the addition of 40 µl of 3.64 mM substrate. After 20 min of further incubation, the 2-napthylamine produced was allowed to undergo a coupling reaction with 100 µl of 10 mM Fast Garnet GBC diazonium salt in the presence of mercurial and detergent Tween 20, and absorbance of the resulting azo dye was measured at 540 nm. The activities of cathepsin B inhibitor were estimated by the following relation: % inhibition=100($A$-$B$)/$A$; $A$ stands for the enzyme activity without inhibitor and $B$ for the enzyme activity with inhibitor. One unit of inhibitor was defined as the amount of inhibitor required for 50% inhibition of 1 unit of cathepsin B.

In the experiment for the inactivation of an inhibitor by proteases, trypsin (26.7 µM) or elastase (121.8 µM) in buffer solution was incubated with an inhibitor in a final volume of 300 µl at 30°C for 23 h. Chymotrypsin (4 µM) was incubated with an inhibitor for 18 h. Each protease was inactivated by heating at 100°C for 5 min to be used as a control. The reaction was stopped by heating at 100°C for 5 min and then the residual inhibitory activity against cathepsin B was assayed. Cathepsin B activity was measured by the same method as that used in kinetic analysis. The final concentration of a substrate was 0.5 mM.

### Kinetic Analysis

For the studies of inhibitor kinetics, the initial velocities of amidase activity of various proteases, including cathepsin B (bovine spleen), papain (papaya latex), trypsin (bovine pancreas), elastase (porcine pancreas), chymotrypsin (bovine pancreas), and proteinase K (Promega Co., U.S.A.), were determined by continuous spectrophotometric assay using various aminoacyl *p*-nitroanilide substrates. *p*-Nitroaniline released from the substrates was monitored at 400 nm at 30°C in a spectrophotometer equipped with a recorder. The absorption coefficient for this substance was known to be 10,500 $M^{-1}cm^{-1}$. Each protease was added to a concentration of approximately 0.01-0.3 µM dissolved in the following buffers: 0.1 M Tris-HCl buffer (pH 7.5) for trypsin, elastase, proteinase K, and chymotrypsin (with 0.05 M $CaCl_2$) and 0.1 M sodium phosphate buffer containing 1.33 mM EDTA (pH 6.0) for cathepsin B (with 2 mM cysteine) and papain (with 5 mM cysteine). Inhibitors were preincubated with enzymes for 10 min and then the reactions were started by the addition of the substrates to a final concentration of 0.1-0.5 mM. The $K_i$ values were determined from Lineweaver-Burk plots [11].

For the transient (pre-steady) state kinetics of inhibitor, the reactions were started by the addition of an inhibitor and a substrate to the enzyme that was preactivated in buffer for 10 min. Reaction progress was measured spectrophotometrically by monitoring the release of *p*-Nitroaniline at 400 nm. Progress curves were composed from 500 to 800 {absorbance, time} pairs. The changes of

enzyme activity during the reaction were calculated from the absorbance intensity.

## RESULTS AND DISCUSSION

### Production of MAPI

*S. chromofuscus* SMF28 was grown in 4 l batches in a 5-l jar fermentor at 30°C. Changes in biomass, concentration of glucose and ammonium ion, and inhibitory activity are shown in Fig. 1. The inhibitory activity increased rapidly during the exponential growth phase reaching maximum value at 43 h culture time, and decreased rapidly thereafter. The production of MAPI was closely related to the mycelium growth. The concentration of glucose decreased rapidly during the exponential growth phase, whereas ammonium ion concentration increased rapidly during the stationary growth phase.

### Purification of MAPI

Th culture broth (12 l) was filtered through GF/C filter paper (Whatman International Ltd., U.K.) to remove mycelia. The resulting filtrate was filtered using ultrafiltration membrane of NMWL 3000 (Millipore Co., U.S.A.). The filtrate was adsorbed onto a columm of Amberlite XAD-7 resin. The column was washed with distilled water and the active substance was eluted with 80% methanol. The active fraction was concentrated and applied to a column of Amberlite IRC-50 ($H^+$ form). The fraction was eluted with 80% methanol containing 0.04 N HCl and concentrated to dryness to give a yellowish syrup. Further purification was carried out using preparative HPLC. The active peak was separated on the JAIGEL-GS310 column (20×500 mm; JAI Co. Ltd., Japan) from the other peaks in 42–56 min, with 40% methanol as the mobile phase at a flow rate of 5 ml/min. The active fraction was further purified by a linear gradient of acetonitrile (24 to 30%) in sodium phosphate buffer (0.02 M, pH 6.0) at a flow rate of 2.5 ml/min on a reverse phase Hypersil H5MOS column (10×250 mm; Hichrom Co., U.K.). The purified inhibitor was freeze-dried and used for further study on the interaction with proteases.

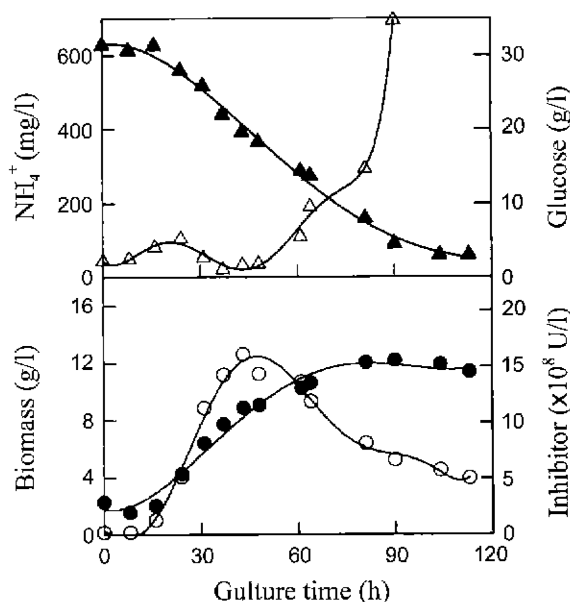


**Fig. 1.** Batch culture data for the production of MAPI from *Streptomyces chromofuscus* SMF28.
The cultures were performed at 30°C with a working volume of 3.0 l, stirred at 300 rpm, and the pH was maintained to 7 0. Aeration was maintained at a rate of 0.5 vvm. Change of biomass (●), MAPI (○), ammonium ion (△), and glucose (▲) were measured

### Identification of MAPI

The purified inhibitor was found to be one of the MAPI group, based on low-resolution FAB-MS analysis (Fig. 2). The fragmentation pattern was similar to that of MAPI isolated from fermentation broths of *Streptomyces* during the screening program for HIV-1 protease inhibitors [19]. The $^1H$ NMR spectra in $CD_3OD$ resolved all proton signals for the proposed structure as MAPI. Stereochemistry of the C-terminal phenylalaninal was not identified in this experiment.

### Kinetic Measurements of the Inhibitory Activity against Proteases

In Table 1, the inhibitory activities of the purified MAPI against various types of proteases were compared. The initial $K_i$ values for chymotrypsin and proteinase K were 0.28 and 0.63 μM, respectively, whereas the values for cathepsin B and papain were 0.66 and 0.28 μM, respectively. Compared with the $K_i$ values of peptide aldehydes such as leupeptin for papain and cathepsin B (2.2 and 6 nM, respectively) [15], chymostatin for chymotrypsin (0.4 nM) [18], and elastatinal for porcine pancreatic elastase (0.24 μM) [22], MAPI is not a potent selective inhibitor for serine or cysteine proteases. In the present experiment, MAPI did not have any inhibitory

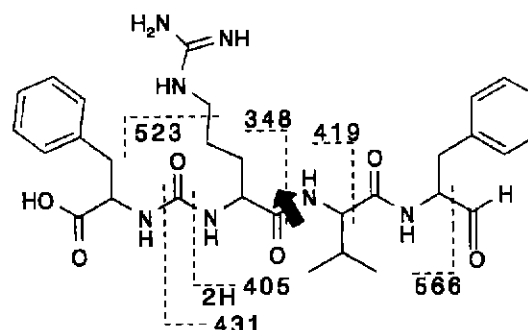


**Fig. 2.** The proposed mass spectral fragmentation pattern for MAPI and the proposed cleavage site (arrow) by trypsin.

**Table 1.** Inhibition of various proteases by MAPI.

| Protease | Substrate | $K_i$ (μM) |
|---|---|---|
| Chymotrypsin | Suc-Ala-Ala-Pro-Phe-pNA | 0.28 |
| Proteinase K | Suc-Ala-Ala-Ala-pNA | 0.63 |
| Cathepsin B | Z-Arg-Arg-pNA | 0.66 |
| Papain | Z-Phe-Arg-pNA | 0.28 |
| Trypsin | Z-Phe-Arg-pNA | NI |
| Elastase | Suc-Ala-Ala-Ala-pNA | NI |

Suc, succinyl; pNA, *p*-nitroanilide: Z, benzyloxycarbonyl.
NI: no inhibition.

activities against trypsin and elastase as previously reported [24].

### Time Course for MAPI Inhibition of Proteases

MAPI exhibited a slow-binding inhibition of cathepsin B activity, evidenced by the progress curve (Fig. 3A). The rate of substrate hydrolysis was decreased from an initial rate to a much slower steady-state rate. In contrast, the inhibitory activity was increased up to a constant value. Slow-binding inhibitors have also been identified for a variety of enzymes, e.g. chymostatin for chymotrypsin and cathepsin G [18], leupeptin for cathepsin B [1], Z-prolinylprolinal for prolyl carboxypeptidase [2], and bestatin for bovine lens leucine aminopeptidase [20].

MAPI was rapidly associated with chymotrypsin before the addition of a substrate. In the presence of a substrate, this enzyme-MAPI complex was partially dissociated and the enzymatic activity gradually increased (Fig. 3B). Thus, the inhibitory activity decreased from 79.3% of the initial value to 45.5% of the final value. Reactivation of MAPI-inhibited enzyme by substrates was also investigated by an experiment of MAPI interaction with membrane bound *Bacillus subtilis* protease [16]. It has been reported that the degree of reactivation of the inhibitor-enzyme complex was dependent on the different substrates.

In the case of proteinase K, the rates of change of substrate hydrolysis were linear and similar with or without inhibitor (Fig. 3C). The Lineweaver-Burk plot gave an intersection on the Y-axis which is characteristic of competitive inhibition.

### Inactivation of MAPI by Proteases

As shown in Fig. 1, the inhibitory activity decreased progressively after a maximum value. It was suggested that proteases might be involved in the inactivation of MAPI, as in the case of leupeptin-inactivating enzyme involved in the inactivation of leupeptin by cleaving the carboxylic side of two leucines [8, 9]. Because MAPI has arginine and valine residues to be cleaved by proteases, trypsin and elastase were chosen to test their hydrolytic

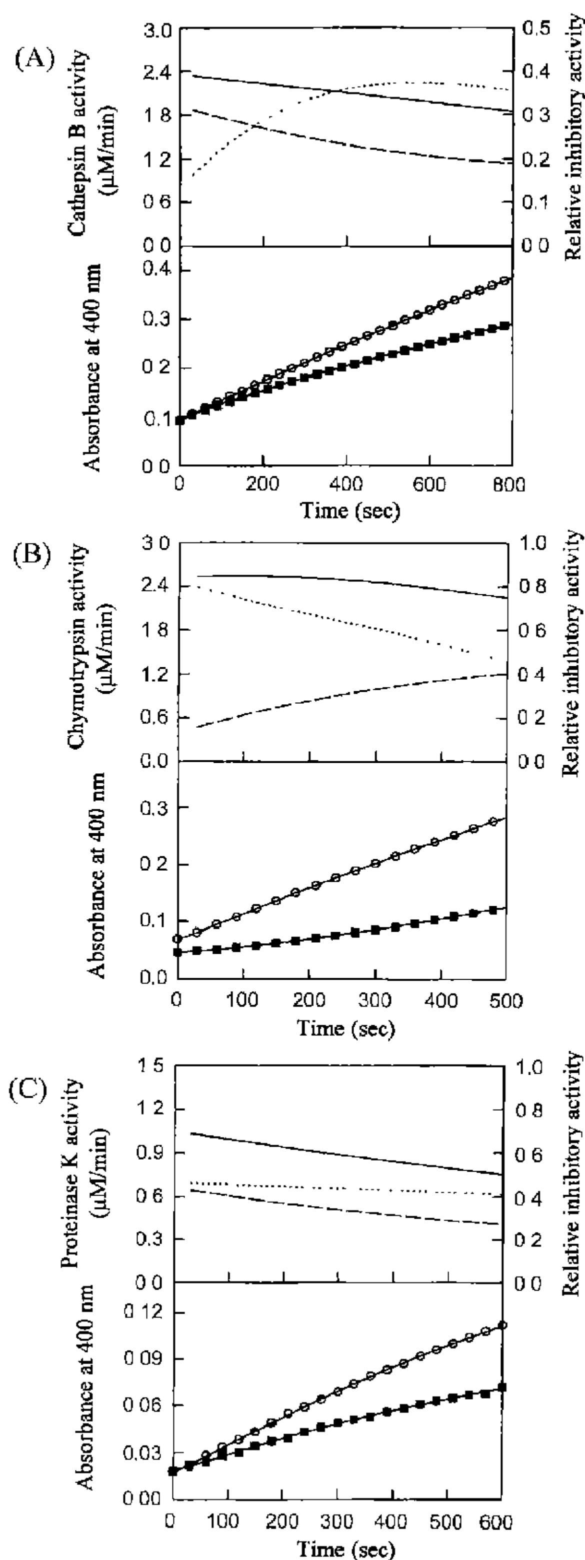


**Fig. 3.** Progress curves for inhibition of cathepsin B (A), chymotrypsin (B), and proteinase K (C) by MAPI.
Reactions were carried out with (■) or without (○) MAPI and monitored every 30 sec. The relative inhibitory activities (- - -) were calculated by the ratio of protease activities with (—) or without (—) MAPI. MAPI was added at the amounts of 386 nM (A), 96.5 nM (B), and 193 nM (C).

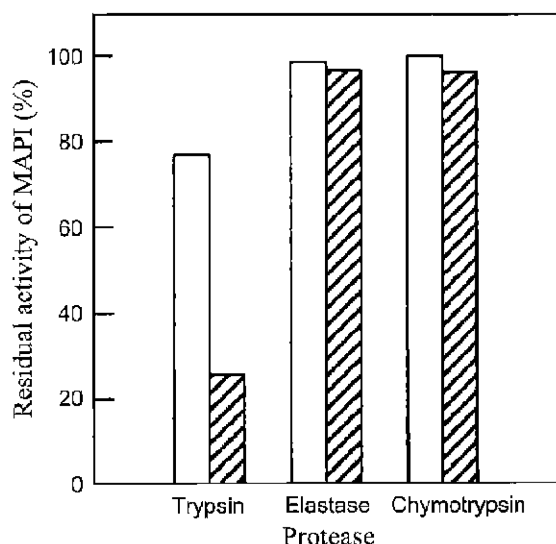


**Fig. 4.** Inactivation of MAPI by proteases.
MAPI was incubated with trypsin (26.7 μM) or elastase (121.8 μM) for 23 h at 30°C and with chymotrypsin (4 M) for 18 h and then the residual inhibitory activity of MAPI was assayed against cathepsin B (hatched bars). The inactive proteases were prepared by heating at 100°C for 5 min and used as controls (open bars).

activity for MAPI, because of their specificity for basic amino acids and uncharged non-aromatic amino acids, respectively [5, 23]. Chymotrypsin was also tested for comparison. The residual inhibitory activities of MAPI incubated with either elastase or chymotrypsin were not changed as compared with the control, indicating that MAPI remained intact (Fig. 4). When MAPI was incubated with trypsin, there was extensive reduction in the inhibitory activity of MAPI corresponding to 66.5% of inactivation of MAPI. This result indicates that a trypsin-like protease may be involved in the decrease of the inhibitory activity during cultivation, and, when MAPI is used as a protease inhibitor in both *in vitro* or *in vivo* experiments, inactivation of MAPI by the trypsin-like protease must be taken into account.

Studies are currently in progress to elucidate the mechanism of inactivation of MAPI by trypsin. The hypothesis that MAPI is cleaved at the carboxylic side of arginine will be initially investigated during analysis of the inactivating-product of MAPI (Fig. 2).

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