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Characterization of Thiol Protease Inhibitor Isolated from *Streptomyces* sp. KIS13

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Streptomyces sp. KIS13 균주에서 분리한 thiol계 단백질분해효소 저해물질의 특성

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***Streptomyces* sp. KIS13 isolated from soil was found to produce low molecular weight thiol protease inhibitors. The protease inhibitor production was closely linked to the cell growth and regulated by growth condition. The inhibitor was purified from the culture broth through butanol extraction, silicagel 60 column chromatography, Sephadex LH-20 gel filtration and preparative HPLC. The inhibitor showed specific inhibitory activity to thiol protease such as papain, picin and bromelain. The mode of inhibition against papain to Hammersten casein as a substrate was non-competitive.**

Since the isolation of leupeptin from the culture broth of microorganisms, a number of protease inhibitors of microbial origin have been found (1-6). The most interesting function of naturally occurring protease inhibitors is that they can control the action of proteolytic enzymes in various tissues and fluid where both the inhibitors and these enzymes are present (4,7-9). Although a large number of low molecular weight protease inhibitors have been found and also their structures have been determined in microorganisms, their biological roles are not clearly elucidated. As a rule, low molecular weight protease inhibitors have an α-amino aldehyde group in the c-terminal part of their peptide molecules and they are biosynthesized by enzymatic process (2,10).

The objective of the current study is to elucidate the biological roles of extracellular low molecular weight protease inhibitor in *Streptomyces* sp. As the first step, *Streptomyces* spp. were isolated from soil and a strain, KIS13, was found to produce extracellular protease inhibitor. Isolation of the inhibitor from the culture broth was attempted and the characters of the purified inhibitor were determined.

Key words: Thiol protease inhibitor, *Streptomyces*
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Materials and Methods

Media used

A rich medium, which was formulated as follows: 1% glucose, 2% peptone, 0.3% yeast extract, 0.3% NaCl, was used for the isolation of Actinomycetes from soil samples. Skim milk was replaced with peptone and yeast extract when the medium was used for the production of extracellular protease. Protease inhibitor producing medium was formulated as follows: 1% glucose, 2% peptone, 0.3% yeast extract, 0.3% NaCl, 0.1% KH_2PO_4, 0.02% $ZnSO_4 \cdot 7H_2O$, 0.002% $FeSO_4 \cdot 7H_2O$, and pH of these media were adjusted to 7.0 before steam sterilization.

Isolation and selection of microorganism producing thiol protease inhibitor

Soil samples were dried in glass petri dishes at room

temperature for 5 days and then heated for 20 minutes at 80°C. The dried soil samples were suspended in distilled water and spreaded on the plates of Actinomycetes isolating meia containing cycloheximide and nystain. Colonies appearing on the plates after 4 days incubation at 30°C were observed and colonies considered to be *Streptomyces* were subjected to the following screening.

The selected colonies were inoculated on the agar pieces of protease inhibitor production medium. And the inoculated agar pieces were incubated for 2 days at 30°C and then transferred to plates of skim milk media where *Streptomyces* sp. producing thiol protease was inoculated at the center of the plate. Colonies inhibiting the formation of clear zone which was resulted from the hydrolysis of skim milk by the proteolytic enzyme were selected as candidates producing protease inhibitors. Colonies selected as candidates to produce protease inhibitor were inoculated to the protease inhibitor producing media which were contained in baffled flasks and cultured 3 days at 30°C using a rotary shaking incubator. The cell free culture broths were filtered through ultrafilters (Amicon YMs; molecular cutting limited 5,000 dalton) and tested for the protease inhibitor activity.

Analytical methods

Cell mass was determined as dried cell weight(DCW) after drying at 80°C for 24 hours. The concentration of glucose was estimated by the dinitrosalicylic acid method (11). The activity of protease was estimated by measuring tyrosine liberated after hydrolysis of Hammersten casein at 37°C and pH 7.5 for 15 minutes. The 1 unit of protease activity was defined as the amount needed for the production of 1 g of tyrosine per minute. The protease inhibitory activity was calculated as follows: % inhibition = 100 (A-B)/A; where, A is the protease activity without inhibitor and B is the protease activity with inhibitor. 1 unit of inhibitory activity was defined as the amount of inhibitor needed for the 50% inhibition of 3.6 unit of papain (Sigma Co.).

The inhibitory activity of the inhibitor on various protease were tested with different substrate as described elsewhere (12,13).

Purification of protease inhibitor

The culture broth was harvested by centrifugation. The protease inhibitor was purified from the culture broth through n-butanol extraction, Silicagel 60 chromatography, Sephadex LH-20 gel filtration and preparative HPLC.

Results and Discussions

Selection of microorganisms producing low molecular weight protease inhibitor

Over 1,000 strains of *Streptomyces* spp. obtained from soil samples were tested to select strains producing low molecular weight protease inhibitors. As a result, an isolant KIS13, was found to produce extracellular low molecular weight protease inhibitor. Fig. 1 showed the typical non-competitive pattern between the protease and the protease inhibitor.

The isolant KIS13 has long rectiflexible spores in chains and spore surface was smooth and color of spore mass was gray. The substrate mycelium was not

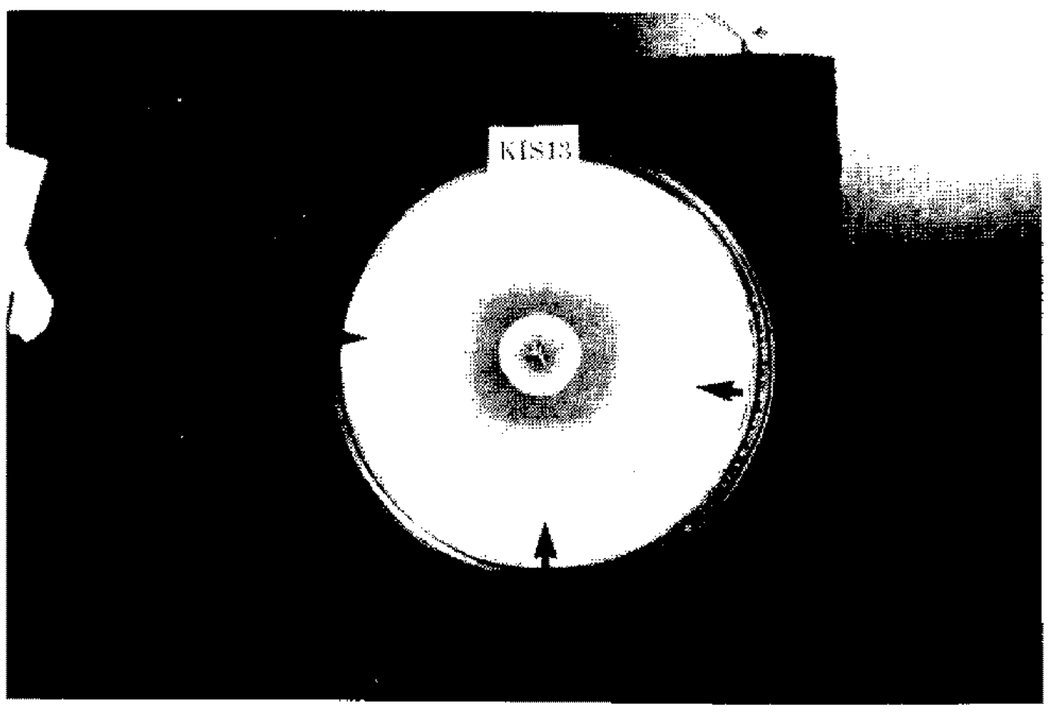

Fig. 1. Antimetabolic pattern between protease and protease inhibitor on skim milk media.
Center colony is *Streptomyces* sp. producing protease and four colonies indicated as (↑) are isolant KIS13

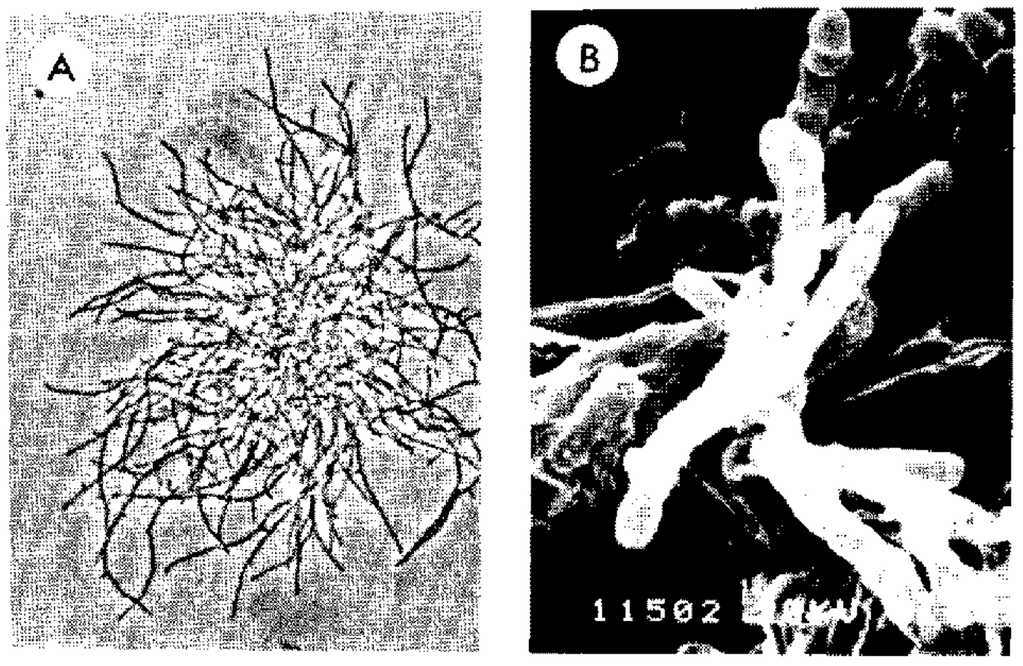

Fig. 2. Morphological characters of an isolant KIS13.
A: Mycelial forms in a submerged culture (×600)
B: Electron microphotography of spore chain (×6.25×10⁴)

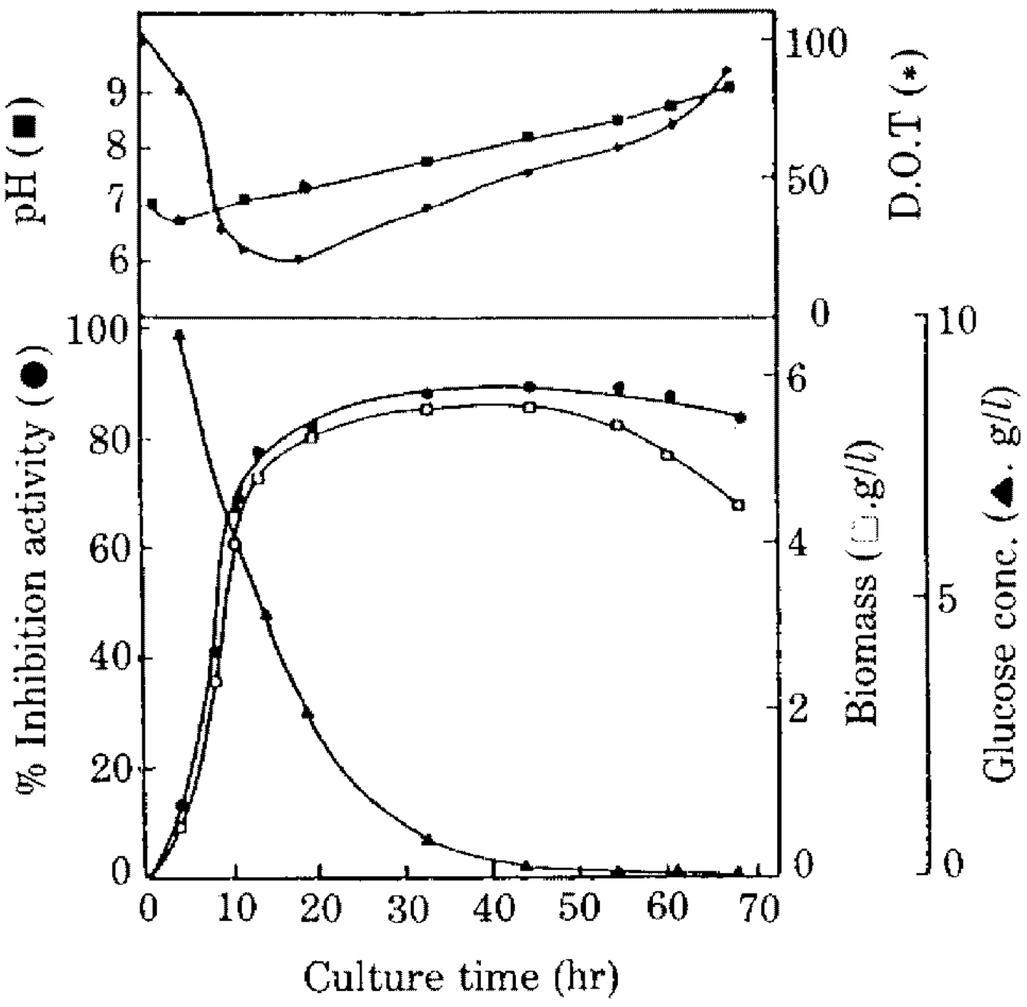

Fig. 3. Changes in the glucose concentration, biomass, protease inhibitor activity, pH and dissolved oxygen tension (D.O.T) in a batch culture.

[The pH and D.O.T were automatically measured by pH electrode (Ingold) and O_2 electrode (Ingold)]

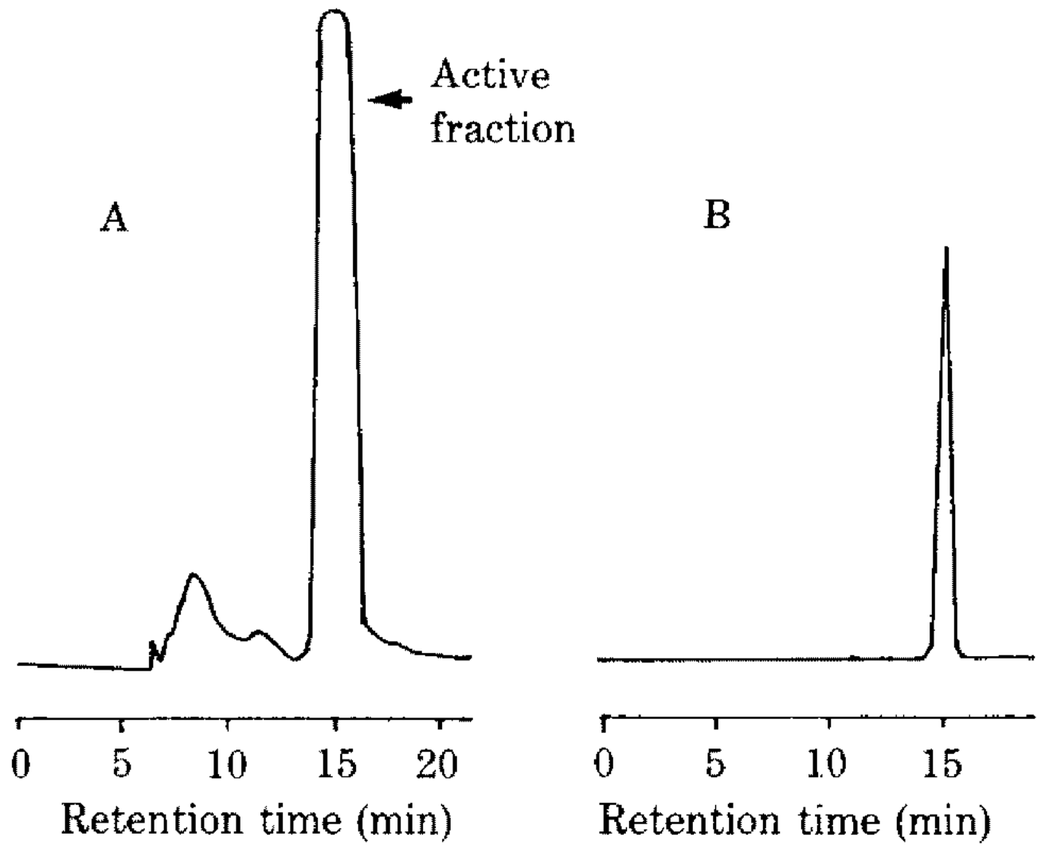

Fig. 4. High-performance liquid chromatography of the protease inhibitor isolated from *Streptomyces* sp. KIS13.

A: Protease inhibitor dissolved in methanol was injected into a lichrosorb RP-18 column for HPLC and eluted with 95% methanol containing 0.1% trifluoroacetate at a flow of 2.0 m*l*/min

B: Active fraction in Fig. 4A was chromatographed again with the same condition

——: Optical density at 235 nm was measured

fragmented. Hence the isolant, KIS13, was tentatively identified as *Streptomyces* sp. (Fig. 2).

Batch culture

Batch culture data for the changes in the concentrations of glucose, biomass, protease inhibitor, pH and dissolved oxygen tension are shown in Fig. 3. The activity of protease inhibitor in the culture broth increased rapidly during the exponential growth phase and gave maximal values at the stationary phase. It was found that the production of protease inhibitor was closely related to the cell growth and the protease inhibitor was a product associated with cell growth. It was worth of noting that disolved oxygen tension was very rapidly reduced as increasing cell mass and also that the cell growth was retarded although the concentration of residual glucose was still very high. The results indicated that other unidentified factor(s) might play important roles on the cell growth and also the inhibitor production.

Purification of protease inhibitor

When the protease inhibitory activity in a batch culture was maxium, the culture broth was harvested by a continuous centrifugation and the culture broth was extracted with n-butanol. The distribution coefficiency of butanol extraction at pH 7 was 0.89 hence about 83.7% of the inhibitor could be recovered from 4 times extraction using half volume of n-butanol. The n-butanol extract was concentrated by vacuum distillation of n-butanol and the residues were dissolved in distilled water.

The butanol extract was applied to a Silicagel 60 column (2.4×46 cm) equilibrated with n-butanol containing methanol (10%). The elution was carried out with same solvent. The flow rate was 30 m*l*/hr and fraction of 10 m*l* was collected. The most active peak (A) of the 4 active peaks designated A,B,C,D was concentrated and applied to a Sephadex LH-20 gel column (2×65 cm) equilibrated with methanol. The elution was carried out with methanol. The flow rate was 5 m*l*/hr and fraction of 3 m*l* was collected. Two major peaks I and II were also found. The major peak II was concentrated and applied to second Silicagel 60 column (2×25 cm) equilibrated with n-butanol. The elution was carried out with n-butanol. The flow rate was 9 m*l*/hr and fraction of 3 m*l* was collected. The active fraction was concentrated and injected into a Lichrosorb RP-18 column for preparative high performance liquid chromatography (prep-HPLC) and eluted with 95% methanol containing 0.1%

trifluoroacetate at a flow rate of 2.0 m*l*/min (Fig. 4). The prep-HPLC consisted of L-5000 LC controller (Hitachi), L4200 UV-VIS detector(Hitachi), L-6000 pump(Hitachi), D-2000 chromato-integrator (Hitachi), and fraction collector(Advantec, SF-139). The purified protease inhibitor obtained from the preparative HPLC was used for the determination of characters.

Characteristics of protease inhibitor

The inhibition spectra of the purified protease inhibitor to various protease was tested. As shown in Table 1, it revealed that the protease inhibitor had inhibitory activity aganist thiol protease *viz* papain, ficin, and bromelain. It was interesting to note that it also inhibited only trypsin from serine protease and acid protease obtained from *Asp. saitoi.*

At present result, the inhibition specificity of the inhibitor was not clear but it was thought that the inhibitor might be synthesized for the specific regulation of protease activity in various physiological processes such as endogeneous metabolism and cell differentiation. In order to know the inhibition mode of the protease inhibitor, the activity of papain on casein was tested with different concentration of protease ibhibitor. The V*max* and K*m* values of the papain on casein were estimated to be 10.1 g tyrosine/min and 2.5 mg/m*l*, respectivity. As shown in Fig. 5, it was clear that the inhibition mode of the protease inhibitor was noncompetitive and the inhibition constant (K*i*) was calculated as 0.124 mg/m*l*. In order to know molecular mechanism of protease and inhibitor, we are determining the structure of protease inhibitor, but we have a certainty that the protease inhibitor inactivate the active thiol group of papain because added cysteine masked the effect of protease inhibitor like iodoacetamide which is the specific alkylating agent of thiol group.

The protease inhibitor was very thermostable to maintain over 93% activity when it was treated at 100°C for 2 hours. It was also stable in wide pH range from 2.0 to 12.0 at 30°C and 60°C. Also, it was founded that the strain KIS13 produced protease instead of protease inhibitor at the other cultural conditions. It was thought that the strain KIS13 has molecular switch mechanism on synthesis either protease or protease inhibitor for regulation of diverse physiological process.

Table 1. Inhibitory spectrum of protease inhibitor produced by *Streptomyces* sp. KIS13 on various proteases.

Protease	Substrate	Inhibition
Thiol protease		
Papain	Casein	+
	PFLNA	+
Ficin	Casein	+
Bromelain	Casein	+
Serine protease		
Trypsin	Casein	+
Chymotrypsin	Casein	−
Pronase E	Casein	−
Subtilish	Casein	−
Metallo protease		
Seratiopeptidase	Casein	−
Acid protease		
Pepsin	Albumin	−
Asp. saitoi	Albumin	+

+; More than 50%.
−; Less than 50%.
PFLNA; p-Glu-Phe-Leu-p-Nitroanilide.

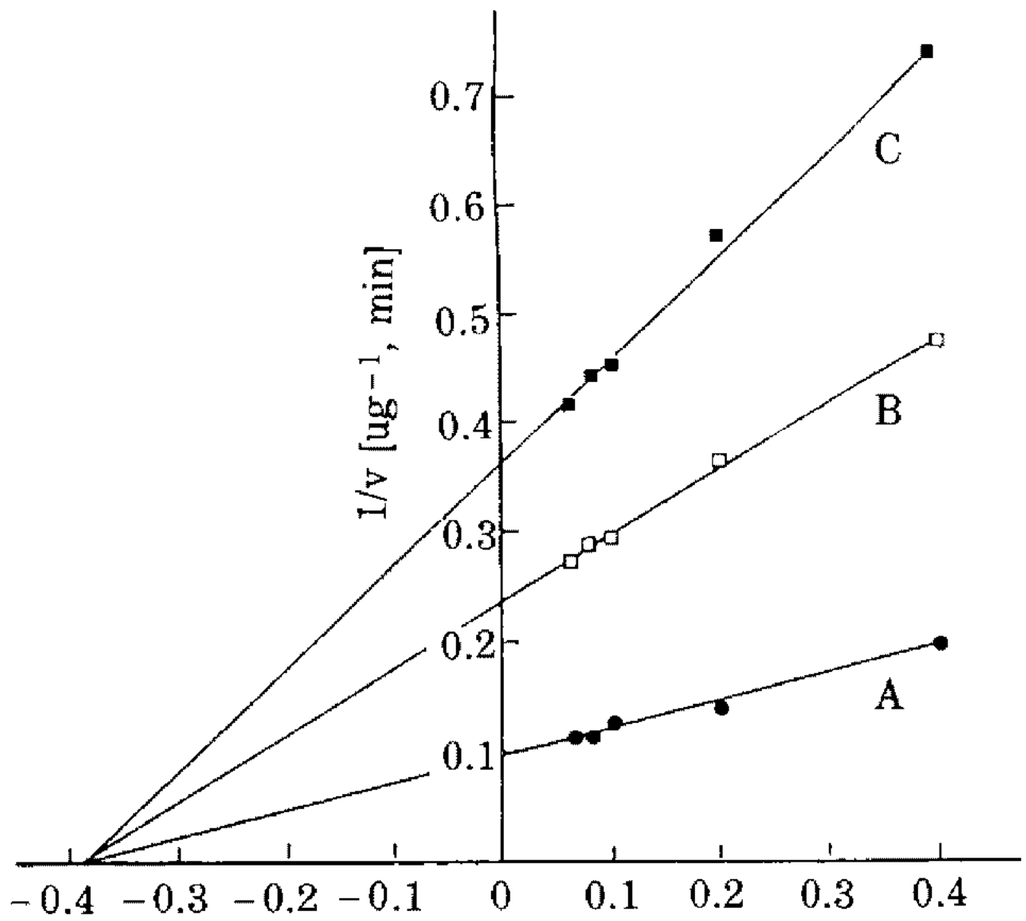

Fig. 5. Type of inhibition against papain by protease inhibitor.

Lineweaver-Burk plots of Hammarsten casein concentration against rate of hydrolysis by papain with and without protease inhibitor
A: in the absence of protease inhibitor
B: in the prosence of protease inhibitor (0.44 mg)
C: in the presence of protease inhibitor (0.88 mg)

요 약

토양으로부터 분리한 *Streptomyces* 속 세균 KIS 13은 thiol 계통 단백질분해효소 활성을 특이적으로 저해하는 저분자량 저해물질을 생성하였다. 저해물질 생성은 균체 성장에 연관된 생성양상을 나타내었다. 배양액으로부터 butanol 추출, silicagel 60 column chromatography, Sephadex LH-20 gel-filtration chromatography, preparative HPLC 등의 과정을 통하여 단백질 분해효소 저해물질을 순수분리하였다. 이 저해물질은 Hammersten casein을 기질로 사용할 때, papain에 대하여 non-competitive한 저해양상을 나타내었다.

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