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A novel extracellular subtilisin inhibitor produced by a *Streptomyces* sp.

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The amino acid composition and inhibitory properties of a protein (SI-1–72) isolated from the culture medium of a *Streptomyces* sp. have been investigated. SI-1–72 appears to be a monomer protein of molecular mass about 13100 Da and amino acid composition which differs from that of the inhibitors of the *Streptomyces* subtilisin inhibitor (SSI) family. Furthermore, it was found to exhibit novel specificity: strong inhibitory effect against microbial alkaline proteinases, moderate effect towards chymotrypsin and elastase, and no inhibition of the other serine proteinases, as well as of the cysteine, aspartate and metallo-proteinases.

Subtilisin inhibitor; Proteinase inhibition; (*Streptomyces* sp.)

1. INTRODUCTION

Natural proteinase inhibitors participate in the control of the cell metabolism by regulating specific proteolytic activation, inactivation and modification of enzymes [1]. This makes them an interesting object for biochemical and clinical studies [2]. A number of extracellular protein inhibitors of microbial alkaline proteinases have been isolated from different streptomycetes [3–6]. Some are classified as members of the *Streptomyces* subtilisin inhibitor (SSI) family on the basis of their common structural features [7]. The active conformation of such inhibitors is ensured by their unique dimer structure [8]. Their inhibitory properties are found to vary: while most of the inhibitors are specific only towards microbial alkaline proteinases [3–5], a number, e.g. plasminostreptin, inhibit plasmin and trypsin as well [6].

In a previous study [9] a protein inhibitor of serine proteinases was isolated in homogeneous form from the culture fluid of *Streptomyces* sp. 1–72 and designated later as a subtilisin inhibitor (SI-1–72). We describe here the amino acid composition and inhibitory properties of this biologically active compound.

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2. MATERIALS AND METHODS

2.1. *Enzymes*

Bovine chymotrypsin, trypsin and thrombin, porcine plasmin and pancreatic elastase, calf rennin and subtilisin Carlsberg were purchased from Sigma (St. Louis, MO); proteinase K, pronase E, papain and porcine pepsin from Merck (Darmstadt); thermolysin, ficin and bromelain from Boehringer (Mannheim); alkaline and neutral proteinases from *Bacillus mesentericus* 90 were kindly provided by Dr D. Kolev (Institute of Microbiology, Sofia); subtilisin DY was prepared as described by Genov et al. [10].

2.2. *Substrates*

Hammarsten casein was obtained from Reanal (Budapest); azocasein from Fluka (Buchs, Switzerland); and *N*-benzoyl-L-arginine *p*-nitroanilide (BAPNA), *N*-benzoyl-L-arginine ethyl ester (BAEE) and *N*-acetyl-L-tyrosine ethyl ester (ATEE) from Sigma.

SI-1–72 was isolated and purified from the culture filtrate of *Streptomyces* sp. 1–72 as in [9].

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The inhibitory effect against caseinolytic activity of serine, cysteine and metallo-proteinases was assayed with azocasein as a substrate [11] and with casein for aspartate proteinases [12]. The inhibition of esterolytic and/or amidase activities of some serine proteinases was determined with the synthetic substrates ATEE, BAEE and BAPNA [11,13]. ID_{50} was graphically calculated according to Umezawa [12].

Amino acid analyses were performed on an LKB 4150 amino acid analyzer after hydrolysis with 6 M HCl for 24 and 72 h. Half-cystine was determined as cysteic acid after oxidation with performic acid. The free thiol content was measured with Ellman reagent [14]. Tryptophan was not determined.

3. RESULTS AND DISCUSSION

SI-1-72 was found to be a protein of molecular mass approx. 12 200 Da as estimated by molecular sieving HPLC and 13 500 Da according to SDS-PAGE. The latter estimate is closer to the value of 13 101 Da calculated from the amino acid composition (table 1). The amino acid composition of SI-1-72 differs from that of the members of SSI family, as shown in table 1. The isolated inhibitor has a relatively greater content of histidine, arginine and glutamic acid, but a lower content of valine and alanine. Titrations with Ellman reagent indicated that no free thiol groups were present. Also of particular interest is the absence of half-cystine residues. In contrast to SSI and other extracellular subtilisin inhibitors from actinomycetes, SI-1-72 obviously does not possess any disulfide bridges, which is characteristic for intracellular proteinase inhibitors isolated from yeast cells [15]. Consequently, SI-1-72 could be regarded as a monomer protein of minimum molecular mass 13 101 Da.

We investigated the inhibitory effect of SI-1-72

Table 1

Comparison of the amino acid composition[a] of SI-1-72 with some other proteinase inhibitors from the SSI family

Amino acid	SI-1-72	SSI[b]	AP-I[b]	API-2b[b]	Plasmino-streptin[b]
Lys	5	2	2	1	3
His	9	2	2	2	2
Arg	10	4	4	4	5
Asp	10	9	9	9	10
Thr	6	8	8	8	11
Ser	8	8	9	10	5
Glu	12	6	6	8	7
Pro	10	8	8	9	5
Gly	11	11	12	11	11
Ala	10	19	19	18	15
½Cys	0	4	4	4	4
Val	7	13	12	13	13
Met	2	3	3	3	2
Ile	3	0	0	1	0
Leu	7	9	9	10	7
Tyr	3	3	3	3	3
Phe	5	3	4	2	5
Trp	–	1	1	1	1
Total	118	113	115	117	109
Molecular mass (Da)	13 101	11 598	11 831	11 872	11 395

[a] Residues per molecule (average values from 24 and 72 h hydrolysates)

[b] Data from [5]

Table 2

Inhibitory effect of SI-1-72 on various serine proteinases

Serine proteinase	Substrate	pH	ID_{50}
Subtilisin Carlsberg	Azocasein	8.8	2.6
	ATEE	8.8	24.8
Subtilisin DY	Azocasein	8.8	2.2
	ATEE	8.8	20.4
B. mesentericus alkaline proteinase	Azocasein	8.8	4.2
	ATEE	8.8	36.4
Proteinase K	Azocasein	8.8	5.4
	BAEE	8.8	16.0
Pronase E	Azocasein	7.4	2.2
	BAEE	7.4	6.8
Bovine chymotrypsin	Azocasein	7.8	21.8
	ATEE	7.8	8.2
Porcine pancreatic elastase	Azocasein	8.8	29.6
Bovine trypsin	Azocasein	7.8	n.i.
	BAEE	7.8	n.i.
	BAPNA	8.0	n.i.
Porcine plasmin	Azocasein	8.8	n.i.
	BAEE	8.8	n.i.
	BAPNA	8.8	n.i.
Bovine thrombin	BAEE	8.8	n.i.
	BAPNA	8.8	n.i.

The concentration of each enzyme except for thrombin was adjusted to the same caseinolytic activity (0.02 Kunitz units in the incubation mixture); the thrombin concentration was 0.6 NIH units. ID_{50}, amount (in μg) necessary for 50% inhibition of activity; n.i., no inhibition

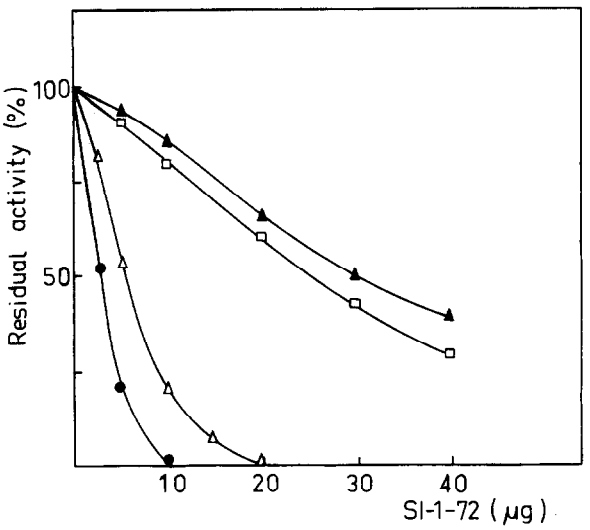

Fig.1. Inhibition of caseinolytic activity of subtilisin Carlsberg (●—●), proteinase K (△—△), chymotrypsin (□—□) and elastase (▲—▲) by varying amounts of SI-1-72. Proteinase activity in the absence of inhibitor is expressed as 100%. Each point represents the average value of 2 assays.

on the caseinolytic activity of various proteinases, namely serine (summarized in table 2), cysteine (papain, ficin and bromelain), aspartate (pepsin and rennin) and metallo-proteinases (thermolysin and *B. mesentericus* neutral proteinase). The inhibitor showed specific inhibitory activity exclusively towards serine proteinases and no effect on the others tested. As shown in table 2, the pattern of inhibition by SI-1-72 includes alkaline proteinases from different microorganisms, chymotrypsin and elastase. Both caseinolytic and esterolytic activities of the investigated enzymes were sensitive to the action of the inhibitor. On the other hand, trypsin, plasmin and thrombin were not inactivated by SI-1-72. Studies on the interaction of the inhibitor with several serine proteinases from different sources established that the degree of inhibition of microbial alkaline proteinases was much greater than that of mammalian proteinases (fig.1). Our data demonstrate an inhibitory specificity which differs from any other reported to date for proteinase inhibitors produced by actinomycetes.

On the basis of its amino acid composition and inhibitory properties, SI-1-72 could be defined as a noval extracellular subtilisin inhibitor from streptomycetes.

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