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CHROMATOGRAPHIC SEPARATION AND PARTIAL CHARACTERIZATION OF MICROBIAL LOW-MOLECULAR-WEIGHT PROTEINASE INHIBITORS

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SUMMARY

A crude preparation, inhibiting trypsin (T), chymotrypsin (C) and papain (P), was isolated from the culture filtrate of *Actinomyces sp.* 9 by butanol extraction. Ion-exchange high-performance liquid chromatography (HPLC) of this preparation on a Mono S column resulted in the separation of two inhibitory fractions: one active against T (TI-9) and the other active against C and P (CPI-9). These two fractions, having the same inhibitory specificity, were also obtained from the crude extract by size-exclusion HPLC on a Superose 12 column.

This method proved to be better in terms of the purity of the fractionated inhibitors than ion-exchange chromatography. Further purification of TI-9 and CPI-9 was achieved by using reversed-phase HPLC on a PEP RPC column. In this case, TI-9 was obtained as an apparently homogeneous peak. Studies on the physicochemical properties of the purified inhibitors showed them to be small molecules, similar in hydrophobicity, pH and thermal stability, but differing in solubility. Amino acid and spectral analyses provided the peptidic structure of TI-9 and gave a molecular weight of 1643, while parallel analyses of CPI-9 showed that it lacks the common amino acids.

INTRODUCTION

Natural inhibitors of proteinases are reported to have been isolated from different mammalian and plant tissues[1–3], as well as from different microorganisms[4–6]. The latter are mostly represented by small peptides[7], in contrast to those found in higher organisms. Recently, microbial proteinase inhibitors have received increased attention because of their potential therapeutic effect in disorders caused by an abnormal elevation of proteolytic activities[8,9]. They are also widely used in biological and biochemical studies[10,11]. The extensive search for low-molecular-weight inhibitors with weak antigenicity resulted in the discovery of a number of novel proteinase inhibitors in the culture filtrates of actinomycetes[12]. Most of them have been purified by sophisticated procedures, involving different extraction and chromatographic methods[13].

In the course of a search for proteinase inhibitors in the fermentation fluids of

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different microorganisms[14], the culture filtrate of *Actinomyces sp.* 9 was found to be effective in inhibiting trypsin (T), chymotrypsin (C) and papain (P). This paper describes the separation of two inhibitory fractions from that source: one active against T (TI-9) and the other active against C and P (CPI-9). The inhibitors were isolated from the culture filtrate by butanol extraction and purified by size-exclusion (SE) and reversed-phase (RP) high-performance liquid chromatography (HPLC). Partial characterization of the purified inhibitors is also described.

EXPERIMENTAL

Materials

Bovine trypsin (Type XIII), bovine α-chymotrypsin (Type VII), papain (Type IV), N-acetyl-L-tyrosine ethyl ester (ATEE), N-α-benzoyl-L-arginine-*p*-nitroanilide (BAPNA) and N-acetyl-D,L-phenylalanine naphthyl ester (APNE) were obtained from Sigma (St. Louis, MO, U.S.A.), Amberlites IR-120, CG-400, XAD-2, and Dowex 50 from Serva (Heidelberg, F.R.G.), Echtblausalz B from Fluka (Buchs, Switzerland) and casein from Reanal (Budapest, Hungary). Antipain, chymostatin and α-microbial alkaline proteinase inhibitor (α-MAPI) were gifts from Dr. W. Maerki, Ciba-Geigy (Basle, Switzerland). Acetonitrile was of HPLC grade; all other chemicals and solvents were of analytical grade.

Actinomyces sp. 9 was grown as previously reported[14]. After removal of the mycelium by filtration, the culture filtrate was boiled for 5 min, centrifuged at 10000 *g* for 10 min and used for investigations.

Methods

Assays for inhibitory activity. The inhibitory effect towards casein hydrolysis by T, C and P was measured according to Umezawa[13], also with the synthetic substrates BAPNA and ATEE[15,16]. HPLC fractions were analysed for T- and C-inhibitory activity by the fast inhibition assay[17], using enzyme-containing agar films.

HPLC. The fast protein liquid chromatography (FPLC) system, was from Pharmacia (Uppsala, Sweden). A Superose 12 HR column (30 cm × 1 cm) was used with 0.05 *M* ammonium acetate (pH 6.5) at a flow-rate of 0.2 ml/min. A Mono S HR column (5 cm × 0.5 cm, Pharmacia) was equilibrated with 0.02 *M* ammonium acetate (pH 4) and eluted with 0.02 *M* ammonium acetate (pH 6), containing 1 *M* sodium chloride, at a flow-rate of 0.7 ml/min. Elution was performed with 30% eluent for 15 min and a linear gradient from 30 to 100% eluent in 15 min. RP-HPLC was performed on a PEP RPC HR column (5 cm × 0.5 cm, Pharmacia) at a flow-rate of 0.5 ml/min. The solvent system comprised 0.05 *M* ammonium acetate (pH 5) (A) and 70% acetonitrile (B) in A. A linear gradient from 0 to 50% B in 30 min was used for elution. All aqueous phases were filtered through MF filters (0.22 μm; Millipore, Bedford, MA, U.S.A.) and degassed under vacuum. The organic solvent was degassed by ultrasonication.

Amino acid analysis. Purified TI-9 and CPI-9 were hydrolysed in 6 *M* hydrochloric acid at 110°C for 48 and 72 h respectively. Amino acid analyses were performed on a Model 4150 amino acid analyser (LKB, Bromma, Sweden). Tryptophan was not determined.

Thin-layer chromatography (TLC). Kieselgel 60 plastic sheets (No. 5748;

Merck, Darmstadt, F.R.G.) were used. The positions of the inhibitors were identified by bioautography on enzyme-containing agar films[17].

RESULTS

Isolation of a crude inhibitory preparation

As reported previously[14], the inhibitors found in the culture filtrate of *Actinomyces sp.* 9 are of low molecular weight. Different methods based on extraction and adsorption were applied to the isolation of a crude inhibitory preparation from the culture filtrate (Table I). As a measure of the efficiency of isolation, the decrease in the inhibitory activity towards C and T in the filtrate after extraction or adsorption was estimated. The results indicate an almost complete extraction of the T- and C-inhibiting compounds by butanol. Preliminary observations showed that butanol and chloroform did not affect the inhibitory properties of the active compounds. The inhibitors were also completely adsorbed on the cation exchangers used, but their recovery was not satisfactory. Therefore, a vacuum-dried butanol extract was used in subsequent studies.

The inhibitory effect of the isolated material was tested against other proteinases, including thiol, metallo- and carboxyl proteinases, and a certain inhibitory activity was detected against papain (data not shown).

Chromatographic separation of two inhibitory fractions from the crude preparation

To analyse the composition of the crude inhibitory material, it was subjected to RP-HPLC on a PEP RPC column, as shown in Fig. 1. Two active fractions were eluted by applying an increasing gradient of acetonitrile, one inhibiting T (TI-9) and the other inhibiting C and P (CPI-9). The chromatogram revealed the complexity of the crude preparation, since many contaminating fractions were also registered.

In order to reduce the number of components present in the crude material, their separation by cation-exchange (CE) and SE-HPLC was examined and com-

TABLE I

COMPARISON OF DIFFERENT METHODS FOR THE ISOLATION OF INHIBITORY PREPARATIONS FROM THE CULTURE FILTRATE OF *ACTINOMYCES SP.* 9

Preparation	*Inhibition* (%) against*	
	Trypsin	*Chymotrypsin*
Culture filtrate	86.1	98.6
After butanol extraction**	14.5	0
After chloroform extraction**	88.4	93.2
After Dowex 50 (H^+)***	0	0
After Amberlite 120 (H^+)***	0	0
After Amberlite CG-400 (Cl^-)***	60.8	19.8
After Amberlite XAD-2***	29.2	18.5

* Inhibition was measured with casein as a substrate (see *Methods*). After extraction or adsorption, the fraction was adjusted to pH 7 and the decrease in the inhibitory activity was estimated.

** Four extractions of 100 ml culture filtrate with 50-ml portions of organic solvent.

*** Portions of 100 ml of culture filtrate were passed through the columns (15 cm × 4 cm).

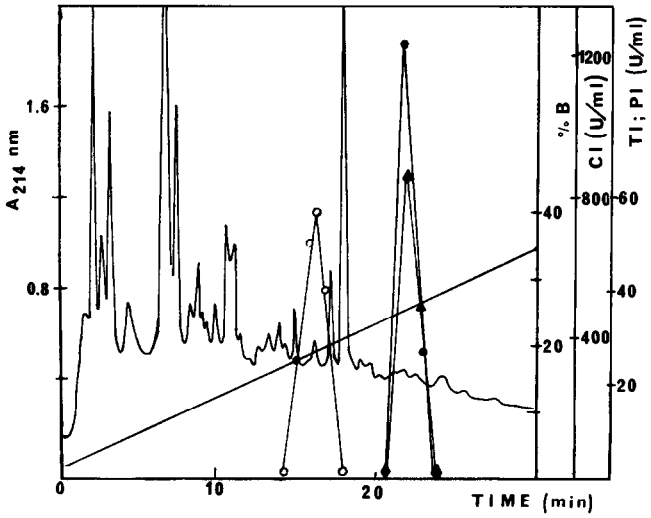

Fig. 1. RP-HPLC of the crude inhibitory preparation. Column: PEP RPC (5 cm × 0.5 cm). Solvents: A, 0.05 *M* ammonium acetate (pH 5); B, 70% acetonitrile in A. Elution with a gradient from 0 to 50% B in 30 min. Flow-rate: 0.5 ml/min. Trypsin inhibition (TI) and papain inhibition (PI) were determined with BAPNA as a substrate, and ATEE was used as a substrate for chymotrypsin inhibition (CI) measurements (see *Methods*). Symbols: O—O, TI; ●—●, CI; ▲—▲, PI.

pared. Fig. 2 shows a clear separation of several fractions by CE-HPLC: the CPI-9-containing fraction was eluted at 0.3 *M* sodium chloride (pH 6), while the TI-9 fraction was desorbed at a higher salt concentration (gradient 0.3–1.0 *M*). The change of pH from 4 to 6 contributed to a more complete desorption of TI-9 from the matrix. Fig. 3 represents SE-HPLC of the crude preparation when the inhibitory fractions were separated from each other and from the other components. To determine the efficiency of purification for both methods used (CE- and SE-HPLC), the active fractions were subjected to RP-HPLC. The elution profiles of the inhibitory fractions separated beforehand by SE-HPLC revealed that they contained less components (Fig. 4a,b). Moreover, under the conditions used (gradient 0–40% B in 30 min), TI-9 was obtained as an apparently homogeneous peak (Fig. 4b).

Characterization of the purified inhibitors

Both inhibitors, TI-9 and CPI-9, were thermostable, since their inhibitory activities were not affected by preliminary boiling of the culture filtrates. Thus, a culture filtrate from *Actinomyces sp.* 9 was assayed for T, C and P inhibitory activity before and after treatment at 95°C for 5 min. The inhibitory activities, measured with casein as a substrate, before and after boiling, were as follows: T inhibition, 58.5 and 56.9 U/ml; C inhibition, 112.3 and 113.4 U/ml; P inhibition, 46.2 and 44.1 U/ml. The active compounds were also stable in the range from pH 2 to 10, monitored by incubations for 20 h at any given pH (data not shown).

The inhibitors were analysed by TLC in acidic and basic solvent systems and their R_F values were compared to those of known microbial peptidic inhibitors, antipain, chymostatin and α-MAPI. As shown in Table II, TI-9 and CPI-9 differ in

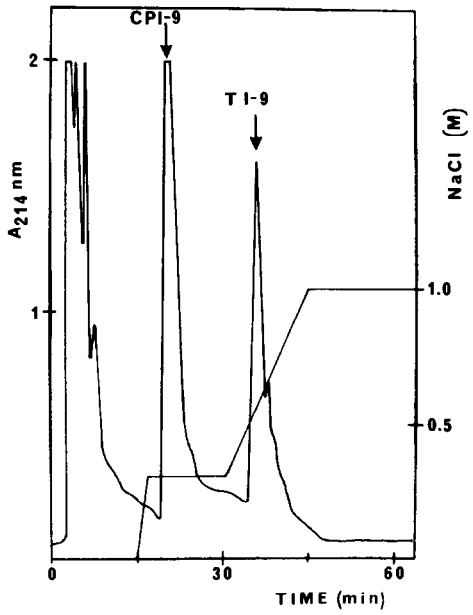

Fig. 2. Separation of the crude inhibitors by CE-HPLC. Column: Mono S (5 cm × 0.5 cm). Solvents: A, 0.02 *M* ammonium acetate (pH 4); B, 0,02 *M* ammonium acetate (pH 6), containing 1 *M* sodium chloride. Elution: (1) with solvent A for 15 min; (2) with 30% B for 15 min; (3) gradient from 30 to 100% B in 15 min. Flow-rate: 0.7 ml/min. PI was determined with BAPNA as a substrate. TI and CI were registere by the rapid inhibition assay (see *Methods*).

their solubilities. TI-9 had a mobility similar to antipain in the acidic system II, but differed in R_F value in the basic system I. On the other hand, CPI-9, chymostatin and α-MAPI demonstrated similar R_F values independently of the system used.

Pronounced differences between the inhibitors isolated were found also with

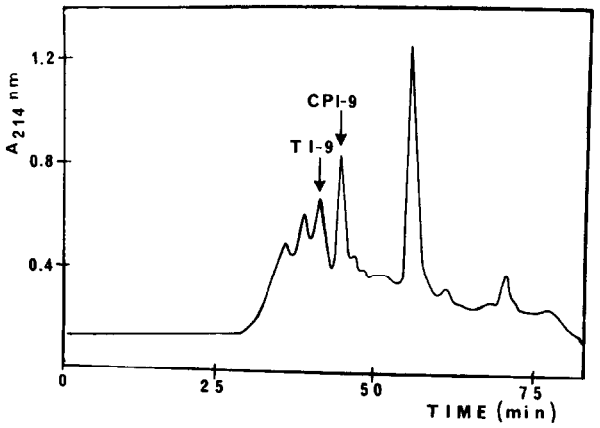

Fig. 3. Separation of the crude inhibitors by SE-HPLC. Column: Superose 12 (30 cm × 1.0 cm). Buffer: 0.05 *M* ammonium acetate (pH 6.5). Flow-rate: 0.2 ml/min. The inhibitory activity was determined as in Fig. 2.

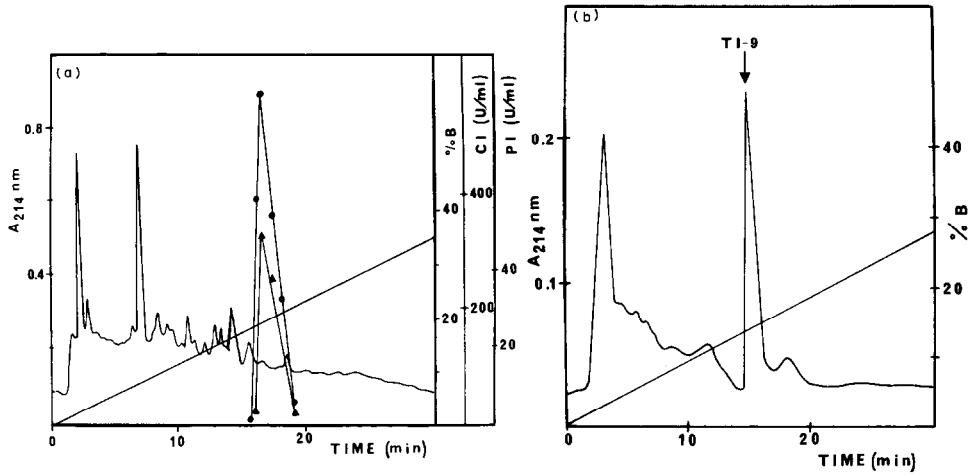

Fig. 4. Final RP-HPLC purification of CPI-9 (a) and TI-9 (b), separated by SE-HPLC. Conditions as in Fig. 1. (a) Sample: CPI-9 fraction from Fig. 3. Elution with a gradient from 0 to 50% B in 30 min. Symbols: ●—●, CI; ▲—▲, PI. (b) Sample: TI-9 fraction from Fig. 3. Elution with a gradient from 0 to 40% B in 30 min.

respect to their ultraviolet absorption spectra and amino acid composition. Thus, in 20% acetonitrile solution, TI-9 exhibited a maximum at 278 nm, while CPI-9 showed no characteristic maximum. The amino acid composition of TI-9 is presented in Table III. The molecular weight of the inhibitor, deduced from the number of amino acid residues, is 1643. The amino acid analysis of CPI-9 revealed the absence of common amino acids.

DISCUSSION

A previous study of the proteinase inhibitors excreted gy *Actinomyces sp.* 9 (ref. 14) confirmed the presence of at least two low-molecular-weight inhibitors, differing in molecular size and specificity. However, the results from the initial extraction or adsorption of these compounds suggested that they have similar chemical prop-

TABLE II

TLC R_F VALUES OF VARIOUS PROTEINASE INHIBITORS

Solvents: I, propanol–ammonia (7:3); II, *n*-butanol–acetic acid–water (4:1:5). TLC was performed on Kieselgel 60 plastic sheets. The positions of the inhibitors were detected by bioautography on enzyme-containing agar films. All numbers given reflect average values with a deviation not exceeding 3–5%.

Inhibitor	*Solvent I*	*Solvent II*
Antipain	0.04	0.25
TI-9	0.39	0.28
CPI-9	0.55	0.63
Chymostatin	0.52	0.63
α-MAPI	0.55	0.62

TABLE III

AMINO ACID COMPOSITION OF TI-9

Amino acid	*Residues per molecule*
Lys	1
Arg	1
Asp	1
Thr	1
Ser	2
Glu	1
Gly	2
Ala	1
Val	1
Ile	1
Leu	1
Tyr	1
Phe	1
Total	15
Molecular weight	1643

erties. Both of them were completely adsorbed on the cation exchangers Dowex 50 and Amberlite IR-120. They were also well extractable by butanol. RP-HPLC of the butanol extract revealed a complex mixture, in which the active compounds seemed to be the minor components. Since the previous attempts to separate the inhibitors by conventional gel chromatography or ion-exchange chromatography were not satisfactory, the higher resolving power of HPLC was applied. A successful separation was achieved by use of both Mono S or Superose 12 columns, but SE-HPLC gave better results in terms of the purity of the inhibitory fractions (TI-9 and CPI-9) isolated. Additional RP-HPLC resulted in further purification of both inhibitors.

The usefulness of RP-HPLC for peptide separations is now widely accepted. There are reports on the use of this technique for final separation and purification of several proteinase inhibitors with similar chemical structures, and characteristics and from the same microbial source[18–20]. The application of HPLC in the present study enabled the successful separation of compounds of unknown nature, which could not be resolved by conventional chromatographic methods. In this case, the application of SE- and RP-HPLC offers an effective procedure for rapid purification of the inhibitors.

The preliminary characterization of the compounds isolated suggested some similarities to the already decribed peptidic inhibitors, such as antipain[21], chymostatin[22] and α-MAPI[18], in their solubility and pH and thermal stability. Furthermore, TLC analyses showed very similar R_F values for CPI-9 and peptides having the same specificity, such as chymostatin and α-MAPI. However, ultaviolet absorption spectra and amino acid analyses of CPI-9 elucidated its quite different nature. Similar analyses of TI-9 revealed its peptidic structure, which differed from previously described small microbial trypsin-inhibiting peptides[12]. At the same time, its intrinsic characteristics (15 amino acid residues per molecule and a molecular weight of 1643) suggested a similarity to the recently reported subtilisin inhibitors, marinostatins, isolated from *Alteromonas sp.*[20].

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