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Isolation and characterization of a protease inhibitor from *Acacia karroo* with a common combining loop and overlapping binding sites for chymotrypsin and trypsin

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ABSTRACT

By using affinity and reversed-phase HPLC (RP-HPLC) chromatographies two chymotrypsin–trypsin inhibitors were isolated from seeds of *Acacia karroo*, a legume of the subfamily *Mimosoideae*. The primary structure of one of these inhibitors, named AkCI/1, was determined. The inhibitor consists of two polypeptide chains, 139 and 44 residues respectively, which are linked by a single disulfide bridge. The amino acid sequence of AkCI/1 is homologous to and showed more than 60% sequence similarity with other protease inhibitors isolated earlier from the group of *Mimosoideae*. AkCI/1 inhibits both chymotrypsin (EC 3.4.21.1) and trypsin (EC 3.4.21.4) in a 1:1 M ratio with K_i values of 2.8×10^{-12} M and 1.87×10^{-12} M, respectively. The P1–P1′ residues for trypsin were identified as Arg68-Ile69 by selective hydrolysis of the inhibitor at this site, with bovine trypsin and human trypsin IV. The cleavage did not affect the inhibition of trypsin, but fully abolished the chymotrypsin inhibitory activity of AkCI/1. This finding together with our studies on competition of the two enzymes for the same combining loop suggests that the same loop has to contain the binding sites for both proteases. The most likely P1 residue of AkCI/1 for chymotrypsin is Tyr67.

Introduction

Since Kunitz discovered a soybean trypsin inhibitor (STI)[1] in 1945 [1], a large number of protease inhibitors, many of them from seeds of the *Leguminosae* (*or Fabaceae*), were isolated and characterized [2–12]. Based on their molecular weights and structural features the *Leguminosae* inhibitors were divided in two groups. One group of these proteins, members of the family I12 (Bowman-Birk family), have about 8 kDa molecular weight and possess high cystine content. The rest of the leguminous protease inhibitors, that STI also belongs to, are members of the family I3 (Kunitz-P family). This group contains proteins with molecular weights of approximately 20 kDa and with a low cystine content [13,14].

The three subfamilies of the family *Leguminosae* are as follows: *Mimosoideae*, *Caesalpinioideae* and *Papilionoideae*. *Acacia* belongs to a subfamily *Mimosoideae*. One of the many species of *Acacia* is *Acacia karroo*, one of South Africa's most beautiful trees, but the genus *Acacia* is no longer indigenous to Africa; therefore, the synonymous name *Vachellia* is also used. The "sweet thorn" has many medicinal uses, ranging from wound poultices to eye treatments and cold remedies. For the first time, we extracted, isolated and throughly characterized from the seeds of *A. karroo* a protease inhibitor that inhibits both chymotrypsin and trypsin at picomolar concentrations. Inhibitors from a few members of the *Acacia* subfamily have already been known to inhibit trypsin and chymotrypsin, though to orders of magnitude different extents [2] (see Table 1). To our knowledge equally strong inhibition of chymotrypsin and trypsin by inhibitors from leguminous seeds, in addition to the present case, has only been reported for an inhibitor from *Leucaena leucocephala* [15]. After the observation of this property of AkCI/1 isolated from *A. karroo*, our research goal has been to identify the combining loop(s) and reactive sites of this inhibitor and to explore the mechanisms of inhibition of the two proteases. Part of this work is reported in this paper.

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[1] *Abbreviations used:* AkCI, *Acacia karroo* chymotrypsin inhibitor; Kunitz-P, plant Kunitz-type inhibitors; RP-HPLC, reversed-phase HPLC; STI, soybean trypsin inhibitor; TCEP, Tris(2 carboxyethyl)phosphine hydrochloride; TFA, trifluoracetic acid.

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Materials and methods

Materials

The *A. karroo* seeds were purchased from Kirstenbosch botanical garden (Cape Town, South Africa) and milled into a fine powder. All of the proteomics grade proteolytic enzymes, synthetic inhibitors and chemicals for amino acid sequencing and inhibition studies were purchased from Sigma–Aldrich (St. Louis, MO, USA). Chemicals for peptide synthesis were products of Applied Biosystems (Foster City, CA, USA) and Merck (Darmstadt, Germany). Recombinant human trypsin 4 was prepared as described by Szilágyi et al. [16].

Preparation of the crude extract

Chymotrypsin inhibitors were extracted from a milled powder of *A. karroo* by a slightly modified method of Joubert et al. [17]. Briefly, 0.15 M NaCl solution was added to 15 g powder to obtain a homogeneous suspension. Another 150 ml of 0.15 M NaCl solution was added to the suspension and was stirred at 0 °C for 3 h. After centrifugation at 5000×*g*, the precipitate was re-extracted as described above. Supernatants were combined and mixed with 740 ml acetone, and kept overnight at 4 °C. The precipitate was separated from the supernatant by centrifugation at 12,000×*g* for 20 min. After vacuum – drying the precipitate, it was dissolved in distilled water and lyophilized. The amount of the lyophilized powder was about 900 mg.

Affinity and RP-HPLC chromatography

The lyophilized extract (15 mg) was dissolved in dH_2O, centrifuged for 4 min at 12,300×*g* by ScanSpeed Mini microcentrifuge (LaboGene, Lynge, Denmark) and the supernatant was subjected to a CNBr-Sepharose 4B chymotrypsin column (3 ml). The column was washed with 5 × 2 ml distilled water and eluted with 7 × 2 ml 50 mM HCl. The fractions exhibiting chymotrypsin inhibitory activity were combined and lyophilized.

N-terminal amino acid sequencing and SDS–PAGE

Purity and the protein content of our AkCI fractions were determined by quantitative N-terminal amino acid sequencing (Edman-degradation), while to estimate the molecular weight and the disulfide bridge structure of AkCI/1 SDS–PAGE was used as described by Laemmli [18] using a 15% w/v polyacrylamide gel in a vertical slab gel apparatus (Bio-Rad, Hercules, CA, USA). Samples were analyzed under reducing (sample buffer contains 5% v/v β-mercaptoetanol) and non-reducing (lack of β-mercaptoethanol) conditions.

Reduction and alkylation

Lyophilized AkCI/1 (50 μg) was dissolved in 0.1 M Tris/HCl (pH 8.5) containing 6 M guanidine hydrochloride. The sample was reduced by addition of 5 μl TCEP (16.5 mg/ml) and incubated for 2 h at room temperature. Then 5 μl N-isopropyliodoacetamide was added for alkylation of the sulfhydryl groups and the reaction mixture was incubated for further 30 min at 37 °C. The alkylated A and B chains were separated on a Vydac C4 RP-HPLC column, then fractions were collected and lyophilized.

Determination of amino acid sequences

All purified AkCI/1 samples, before they were used for further studies, were subjected to quantitative N-terminal amino acid sequencing up to the 5th–7th amino acid residues. Lyophilized fractions of the reduced and alkylated AkCI/1 were dissolved in 20 μl 0.1 M NH_4HCO_3 (pH 8.0). Samples were separately digested with 0.2 μg bovine trypsin, bovine chymotrypsin and *Staphylococcus aureus* V8 protease at 37 °C for 30 min. Reactions were stopped by adding 2 μl 10% v/v TFA to the solutions. Each digest was lyophilized, redissolved in 0.1% v/v TFA and separated by RP-HPLC on a C18 Brownlee capillary column (Perkin Elmer, MA, USA). The amino acid sequences of the separated peptides were determined by automated N-terminal Edman degradation – using an ABI Procise 494 protein sequencer (Applied Biosystems Inc., Foster City, CA, USA) – and by mass spectrometry.

Mass spectrometry analyses were performed on a Bruker Esquire 3000+ ion trap mass spectrometer (Bruker, Bremen, Germany) equipped with an electrospray ionization (ESI) source. Spray voltage was set to 4.0 kV. Samples were dissolved in a mixture of acetonitrile/water (1:1 v/v) containing 0.1% acetic acid and introduced by a syringe pump with a flow rate of 10 μl/min. Ions were detected in positive mode in the range of 50–3000 *m*/*z* with 13.000 *m*/*z*/s scan resolution.

Determination of the inhibitory constant (K_i) values

Bovine trypsin and chymotrypsin stock solutions were made by dissolving the proteins in 10 mM HCl containing 10 mM $CaCl_2$. Active enzyme concentration was determined by active-site titration as described by Jameson et al. [19] using a fluorescent burst titrant 4-methylumbelliferyl p-guanidinobenzoate. The FluoroMAX™ spectrofluorometer (SPEX Industries, Edison, NJ, USA) was calibrated with methylumbelliferone. The active inhibitor concentration was determined as described by Szenthe et al. [20] using bovine trypsin in 50 mM Tris/HCl (pH 8.0) containing 10 mM $CaCl_2$ and 0.005% v/v Triton X-100 reaction buffer. The enzyme substrate was Nα-Benzoyl-DL-arginine p-nitroanilide hydrochloride.

The equilibrium inhibitory constants (K_i) were determined by the method of Empie and Laskowski [21] as described in detail

Table 1
Tentative binding sites (P3–P3′) for trypsin and chymotrypsin in a few protease inhibitors from leguminous seeds.

Origin of inhibitor	Trypsin	Chymotrypsin	Refs.
Glycine max (Soybean)	***61*PYR IRF*66***	***60*SPY RIR*65***	Koide and Ikenaka [27], this work
Acacia karroo	***66*PYR IAI*71***	***65*TPY RIA*70***	This work
Leucaena leucocephala	*62*PYR ILI*67*	*61*SPY RIL*66*	Oliva et al. [15]
Acacia confusa	***62*PPK IAI*67***	*66*AIL TPA*71*	Hung et al. [37]
Enterolobium contortisiliquum	***62*PPR IAI*67***	*66*AIL TPA*71*	Batista et al. [10]
Prosopis juliflora	***62*PPR IAI*67***		Negreiros et al. [9]
Acacia schweinfurthii	*64*PPR IAF69		Odei-Addo et al. [12]
Adenanthera pavonia	***62*PPR IRY*67***		Macedo et al. [38]

Strong binding ($K_i = \sim 10^{-10-12}$ M, marked with bold letters); relatively weak binding ($K_i \leqslant \sim 10^{-9}$ M).