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Immobilized analogues of sunflower trypsin inhibitor-1 constitute a versatile group of affinity sorbents for selective isolation of serine proteases

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ABSTRACT

Sunflower trypsin inhibitor-1 (SFTI-1), a natural 14-residue cyclic peptide, and some of its synthetic acyclic variants are potent protease inhibitors displaying peculiar inhibitory profiles. Here we describe the synthesis and use of affinity sorbents prepared by coupling SFTI-1 analogues to agarose resin. Chymotrypsin- and trypsin-like proteases could then be selectively isolated from pancreatin; similarly, other proteases were obtained from distinct biological sources. The binding capacity of [Lys5]-SFTI-1-agarose for trypsin was estimated at over 10 mg/mL of packed gel. SFTI-1-based resins could find application either to improve the performance of current purification protocols or as novel protease-discovery tools in different areas of biological investigation.

1. Introduction

SFTI-1 is a natural 14-residue homodetic cyclic peptide, cyclo(-Gly-Arg-Cys-Thr-Lys-Ser-Ile-Pro-Pro-Ile-Cys-Phe-Pro-Asp-) bisected by a disulfide bridge, which was isolated from seeds of *Helianthus annuus* as a potent inhibitor of trypsin activity, with a sub-nanomolar K_i value [1]. In spite of the sequence and structural similarities between SFTI-1 and the reactive loop of the Bowman–Birk protease inhibitors, there is no further resemblance between the remainder of the precursor protein from which the cyclic SFTI-1 peptide is excised and the known Bowman–Birk inhibitors [2]. SFTI-1 is amenable to chemical synthesis [3] and some active analogues have been prepared by substituting an appropriate amino acid residue for the naturally occurring Lys at position 5 of the inhibitors [4–6]. SFTI-1 or its synthetic analogues were capable of inhibiting, with variable levels of specificity, serine proteases such as trypsin [1], chymotrypsin [7], elastase [8] and matriptase [3]. Furin also is a likely target for SFTI-1 variants [9]. An advantageous feature of the SFTI-1 scaffold for generating selective tools both for biochemical investigation of proteolytic enzymes and for treatment of serine protease-associated diseases [3,9] is the relative easiness with which synthetic SFTI-1 variants can be optimized for inhibiting a particular protease [8]. Moreover, the disulfide-bridged Gly-Arg-Cys-Thr-Lys-Ser-Ile-Pro-Pro-Ile-Cys-Phe-Pro-Asp acyclic SFTI-1 analogue is nearly as active as the natural homodetic cyclic peptide [10], thereby allowing for the creation of novel inhibitory specificities by conventional chemical synthesis without the need for preparing cyclic peptides. Thus, SFTI-1 and its synthetic analogues are potential alternatives to naturally occurring protease inhibitors of plant and animal sources as ligands for the affinity chromatographic isolation of serine proteases. Although distinct natural inhibitors have long been used for this purpose [11,12], only group-specific affinity media could be generated by covalent attachment of any such inhibitor to a suitable solid support because most of these inhibitors bind serine proteases regardless of the proteolytic specificities of the enzymes. This lack of binding selectivity has been overcome, in some instances, by using selective elution protocols of the affinity column [13] or, else, by tandem affinity chromatography using orderly connected columns prepared with different inhibitors [14]. Novel or even peculiar inhibitory specificities have been ascribed to some synthetic protease inhibitor analogues [4–6,15] and to mutated recombinant forms of natural inhibitors [16,17]; however, the development of more specific and selective affinity chromatographic media that takes advantage of the inhibitory properties of these compounds has yet to be demonstrated.

Here we report the development of an effective means to covalently attach synthetic acyclic SFTI-1 analogues to porous agarose support while retaining their inhibitory activities and specificities, and describe the preparation of selective affinity resins and their use for the isolation of serine proteases from various biological sources.

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2. Experimental

2.1. Materials

A mixture of swine pancreatic enzymes, or pancreatin, was obtained as the breakthrough fraction of a Sephadex G-25 column (1.5 × 10 cm) loaded with 1.0 mL of the supernatant solution (1000 *g* × 5 min) of a crushed Pankreoflat® tablet dissolved in 5.0 mL of ice-cold 50 mM sodium acetate buffer, pH 4.5.

Pankreoflat® (Solvay Pharmaceuticals, Germany), a worldwide available medicine, was purchased at the local drugstore. The rat mesenteric arterial bed perfusate and rat elastase-2 were obtained as previously described [18]. All animal protocols used in this work were approved by the Faculty of Medicine of Ribeirão Preto Institutional Animal Care and Use Committees. Dried *Bothrops moojeni* snake venom was supplied by the FMRP Serpentarium, São Paulo University. Bovine trypsin (224 μm/mg) and alpha-chymotrypsin (55 μm/mg) were from Worthington Biochemical Co., N.J, USA. The chromogenic substrates *N*-benzoyl-DL-Arg-*p*-nitroanilide (DL-BA*p*NA), *N*-succinyl-Ala-Ala-Pro-Phe-*p*NA (*N*-succinyl-AAPF-*p*NA), *N*-succinyl-Ala-Ala-Pro-Leu-*p*NA (*N*-succinyl-AAPL-*p*NA) and *N*-succinyl-Ala-Ala-Ala-*p*NA (*N*-succinyl-AAA-*p*NA), marketed as substrates for trypsin, chymotrypsin, elastase-2 and elastase, respectively, were from Sigma Chemical Co., MO, USA. Angiotensins I and II (Ang I and Ang II), dimethylformamide, trifluoroacetic acid (TFA), 1,4-butanediol diglycidyl ether, hydrazine hydrate 85% (w/v), Sepharose 6B, 6-aminocaproic acid and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDAC) were purchased from Sigma–Aldrich.com.br. Rink resin (0.7 mmol/g), *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium-hexafluoro-phosphate (HBTU), 1-hydroxybenzotriazole hydrate (HOBt), Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Pro-OH, Fmoc-Phe-OH, Fmoc-Ile-OH, Fmoc-Leu-OH and Fmoc-Asp(OtBu)-OH were from Advanced ChemTech, KY, USA. C-18 Sep-Pak cartridges were from Millipore Co., MA, USA. Hydrazide–agarose (approx. 1.0 μmol/mL of packed gel) was obtained by reacting 6-aminocaproic acid-Sepharose 6B, prepared by the bis-oxirane coupling procedure [19], with equal volume of 1.2 M hydrazine-Cl, pH 5.0, in the presence of 0.1 M EDAC overnight. Analytical and small scale preparative HPLC separations were carried out on a Shimadzu HPLC system composed of system controller SCL 6B, auto injector SIL-6B, two pumps LC-6AD and UV–vis spectrophotometric detector SPD-6AV; the detector analogic signal was digitalized by a DI-191 RS waveform recording system (Dataq Instruments, OH, USA) and chromatograms generated by using a WinDaq/Lite Chart Recorder software.

2.2. Enzyme assays

All assays were carried out at 37 °C by incubating the specified substrate with the indicated enzyme in 0.03 M Tris–HCl buffer, pH 8.1, containing 0.15 M NaCl. The hydrolysis rates of chromogenic substrates were determined by recording the time-dependent increase in $A_{410\,nm}$ from the liberation of *p*-nitroaniline [20]. Enzyme-catalyzed cleavage of Ang I was measured by reversed-phase HPLC analysis of peptide fragments as described previously [18], on a Shimadzu SCL-6B equipment fitted with a 4 mm × 250 mm Shim-pak ODS column; peptides were eluted with a linear gradient of acetonitrile concentration (12–42%, v/v; 30 min) in 0.1% (v/v) TFA, at a flow rate of 1.0 mL/min, and monitored by absorbance at 215 nm. Ang I and its fragments were identified by the corresponding retention times and quantified using cognate synthetic peptides as standards. Plasma clotting activities of *B. moojeni* venom and fractions thereof were assayed by a modification of the method described [21] by incubating 0.2 mL of heparinized rat plasma with 20 μL of serially 2-fold diluted enzyme samples in Tris buffered saline; activities were then determined based on clot formation and expressed as the reciprocal of the clotting titer as read at 5 min of incubation time.

2.3. Determination of IC_{50} and K_i

IC_{50} values, the inhibitor concentrations at which the enzyme reaction rates are 50% of the respective uninhibited reactions, were obtained by nonlinear regression analysis (GraphPad Prism Software) of inhibition curves performed, in duplicates, using the indicated SFTI-1 analogues in concentrations ranging from 40 pM to 25 μM. The inhibition constants K_i were derived from the respective IC_{50} values using the Cheng and Prusoff equation [22].

2.4. Peptide synthesis

Each of the SFTI-1 analogues was manually assembled on 100 mg of Rink resin using the HBTU/HOBt activation protocol for Fmoc solid-phase peptide synthesis [23]. Disulfide bond formation was achieved by the DMSO mediated oxidation of free thiols, as previously described for SFTI-1 analogues [7]. Peptides were purified by HPLC using a C-18 reversed phase column (Vydac 218TP5415; 4.5 cm × 15 cm) developed at a flow rate of 1.0 mL/min with a linear gradient of acetonitrile concentration (6–36%, v/v; 30 min) in 0.05% (v/v) TFA. Samples of about 1.0 mg of crude peptide in 1.0 mL 0.05% (v/v) TFA were individually processed, yielding 300–450 μg of purified peptide per run. Under these conditions the retention times for the peptides were: [Arg5]-SFTI-1, 18 min; [Lys5]-SFTI-1, 21 min; [Leu5]-SFTI-1, 22 min; and [Phe5]-SFTI-1, 24 min. Amino acid composition and concentration of the purified peptides were determined based on amino acid analysis after acid hydrolysis.

2.5. Synthesis of SFTI-1-based supports and affinity chromatography

Site-directed conjugation of SFTI-1 analogues to hydrazide–agarose was performed using the rationale that N-terminal Ser residues of peptides are readily oxidizable by periodate to create an aldehyde function, which can then be coupled to hydrazides via stable hydrazone linkages [24]. Briefly, 0.3 mM solutions of SFTI-1 analogues bearing an additional N-terminal Ser residue were prepared in 2.0 mL of 0.1 M sodium phosphate buffer, pH 6.0, and incubated with 3.5 mM $NaIO_4$ for 60 min at room temperature in the dark; the corresponding carbonyl-containing peptides formed were recovered by Sep-Pak extraction and dried under vacuum. Coupling was carried out by incubating 2.0 mL of 0.7 mM of each carbonyl-SFTI-1 analogue in sodium phosphate buffer, pH 6.0, with 2.0 mL of hydrazide–agarose overnight at 4 °C. Samples of about 0.5 mL of individual proteolytic mixtures, prepared in 0.1 M Tris-buffered isotonic saline, pH 8.1, were percolated by gravity at room temperature through a 6 mm bore plastic column packed with 0.6 mL of the indicated affinity resin; after thorough washing of the respective column with buffered saline to remove loosely bound proteins, specifically bound proteases were eluted with 20 mM HCl, care being taken to neutralize any acid excess in the fractions just after collection.

2.6. SDS-PAGE analysis

Proteins were separated by SDS-PAGE on polyacrylamide gels under reducing [25] or non-reducing conditions using a BioRad MiniProtean III system. Molecular mass standard proteins used were bovine serum albumin (67 kDa), carbonic anhydrase (29 kDa)