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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)

Table 1
K_i values for the inhibition of trypsin, chymotrypsin and elastase-2 by some acyclic SFTI-1 analogues.

Inhibitor[a]	Bovine trypsin	Bovine chymotrypsin	Rat elastase-2
[Lys5]-SFTI-1	$(1.0 \pm 0.2) \times 10^{-10}$ M[b]	No inhibition[c]	No inhibition[c]
[Arg5]-SFTI-1	$(1.0 \pm 0.0) \times 10^{-8}$ M[d]	$(4.4 \pm 0.2) \times 10^{-7}$ M[e]	No inhibition[c]
[Phe5]-SFTI-1	$(2.2 \pm 0.0) \times 10^{-7}$ M[f]	$(5.0 \pm 0.1) \times 10^{-10}$ M[g]	$(3.4 \pm 0.1) \times 10^{-7}$ M[h]
[Leu5]-SFTI-1	No inhibition[c]	$(1.0 \pm 0.1) \times 10^{-7}$ M[i]	$(1.2 \pm 0.1) \times 10^{-7}$ M[j]

[a] General structure: Gly^1-Arg^2-Cys^3-Thr^4-Aaa^5-Ser^6-Ile^7-Pro^8-Pro^9-Ile^{10}-Cys^{11}-Phe^{12}-Pro^{13}-Asp^{14}. Inhibitors are identified by the specified residue at position 5. Cys^3 and Cys^{11} are disulfide linked.
[b] Based on equilibrium association constant value of $9.9 \pm 0.7 \times 10^9$ M^{-1} [10].
[c] No inhibition was observed at or below 25 μM concentration of this SFTI-1 analogue.
[d] Based on IC_{50} values of $(3.3 \pm 0.0) \times 10^{-8}$ M using the Cheng and Prusoff equation [22].
[e] Based on IC_{50} values of $(9.7 \pm 0.5) \times 10^{-7}$ M using the Cheng and Prusoff equation [22].
[f] Based on IC_{50} values of $(3.7 \pm 0.0) \times 10^{-7}$ M (this work).
[g] Based on equilibrium association constant value of $2.0 \pm 0.1 \times 10^9$ M^{-1} [6].
[h] Based on IC_{50} values of $(5.9 \pm 0.1) \times 10^{-7}$ M using the Cheng and Prusoff equation [22].
[i] Based on IC_{50} values of $(1.1 \pm 0.1) \times 10^{-7}$ M using the Cheng and Prusoff equation [22].
[j] Based on IC_{50} values of $(2.2 \pm 0.1) \times 10^{-7}$ M (this work).

and lysozyme (14.3 kDa). Gels were stained with Coomassie blue R-250 (0.25%, w/v, in 7%, v/v acetic acid–45%, v/v, ethanol) for 30 min and destained in 10% (v/v) acetic acid–5% (v/v) ethanol.

3. Results and discussion

Affinity resins prepared with naturally occurring serine protease inhibitors as ligands constitute important chromatographic tools for the isolation of proteases [14,26–28]. They have, however, limited proteolytic selectivities reminiscent of the inhibitory specificities of the respective immobilized ligands. In recent years, however, the development of SFTI-1 analogues with altered specificities [6–8] has supplied researchers with a potentially versatile source of ligands to create selective affinity supports for the purification of serine proteases. Indeed, the data of Table 1 show that the inhibitory specificities of these analogues correlate with the occupancy of position five of their sequences by different amino acids, as indicated by the K_i values of four such analogues for trypsin, chymotrypsin and elastase-2. Thus, to investigate whether immobilization of different ligands with the SFTI-1 scaffold would yield affinity supports of restricted specificities for the isolation of proteases, we prepared and tested the respective affinity resins. Since the Gly^1-Asp^{14} acyclic version of SFTI-1 was shown to be nearly as active as the naturally occurring 14-mer cyclic homodetic peptide SFTI-1 [10,29], all affinity resins were prepared in this work with acyclic peptides which, from a practical standpoint, have advantages compared with analogues with a cyclized backbone bisected by a disulfide bond. First, inhibitors can be conveniently assembled by standard peptide synthesis protocols and, second, a reactive carbonyl group can be generated by gentle periodate oxidation of an additional N-terminal serine residue, which ensures a site-directed coupling of the inhibitors to hydrazide–agarose [24]. The affinity resins thus prepared using the acyclic analogues [Lys5]-SFTI-1, [Phe5]-SFTI-1, [Arg5]-SFTI-1 and [Leu5]-SFTI-1 as ligands yielded efficient chromatographic media for the isolation of serine peptidases (Figs. 1–3). The selectivities of these resins toward proteases of different biological sources were reminiscent of the K_i values for the inhibition of trypsin, chymotrypsin and elastase-2 by the respective SFTI-1 analogue (Table 1).

The results of Fig. 1A and B allow direct comparison between the chromatographic selectivities of immobilized [Lys5]-SFTI-1 and [Phe5]-SFTI-1 toward pancreatin proteases. Thus, samples containing nonsaturating amounts of pancreatin chromatographed over either [Lys5]-SFTI-1-agarose or [Phe5]-SFTI-1-agarose resulted in distinct elution profiles, whereby all of the trypsin-like enzymes of the pancreatin, revealed by their BA*p*NA-hydrolyzing activities, were retained by the former column and then recovered as an enzymatic preparation essentially free of chymotrypsin- and elastase-like enzymes; on the other hand, the [Phe5]-SFTI-1-agarose column retained part of the *N*-succinyl-AAPF-*p*NA-hydrolyzing activity of the applied sample, which was subsequentially recovered as a chymotrypsin-like preparation devoid of other pancreatin serine proteases such as trypsin and elastase, the latter being identified by the cleavage of the chromogenic substrate *N*-succinyl-AAA-*p*NA. Since pancreatin contains elastase-2, a protease that also cleaves the substrate *N*-succinyl-AAPF-*p*NA [20], it is possible that the [Phe5]-SFTI-1-agarose resin be able to discriminate between dif-

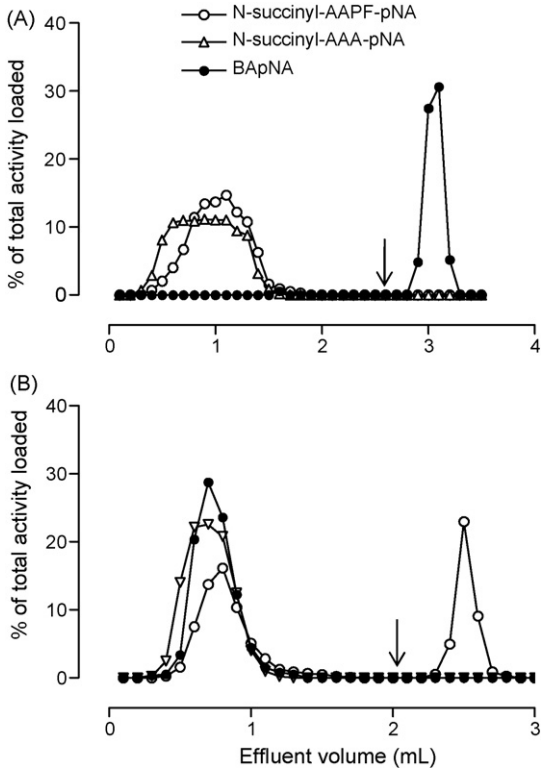

Fig. 1. Affinity isolation of pancreatic proteases using immobilized SFTI-1 analogues. Samples of porcine pancreatin, containing non-saturating amounts of proteases, were loaded onto columns packed with either [Lys5]-SFTI-1-agarose (A) or [Phe5]-SFTI-1-agarose (B) resins equilibrated with 50 mM Tris buffer pH 8.1 containing 0.15 M NaCl or 0.5 M NaCl, respectively. Specifically bound proteins were eluted from each column with 20 mM HCl (arrow). Aliquots of the collected fractions were assayed for proteolytic activity toward the indicated chromogenic substrates.

ferent *N*-succinyl-AAPF-*p*NA-hydrolyzing enzymes to account for the corresponding activity that eluted in the breakthrough volume of the column (Fig. 1B). Overall, the combined results of Fig. 1A and B clearly show that a single-residue modification in the SFTI-1 moiety drastically changed the binding selectivity of the corresponding affinity support, consistently with the inhibitory profiles of the [Lys5]-SFTI-1 and [Phe5]-SFTI-1 analogues shown in Table 1; also, the results suggest that the specificities and selectivities of affinity sorbents based on SFTI-1 analogues are amenable to fine-tuning by judicious selection of chemically modified ligands. It is worth mentioning that the conditions used during chromatography are also determinants of the selectivity of the affinity resins, as illustrated in Fig. 1B, whereby addition of 0.5 M NaCl to the sample buffer was required to fully eliminate the recovery of BA*p*NA-hydrolyzing activity from among those displayed by the enzymes that specifically bound the [Phe5]-SFTI-1-agarose. It has already been shown that functionally distinct SFTI-1 analogues can be created by chemical synthesis following either combinatorial library [6,30], substitutional analysis [8] or rational design [7] approaches. Such inhibitory diversity has been attained by modification of the SFTI-1 moiety not only at position 5, which supposedly determines the primary inhibitory specificity, but also at other positions along the SFTI-1 sequence. The double mutated SFTI-1 analogue V2R/L5K

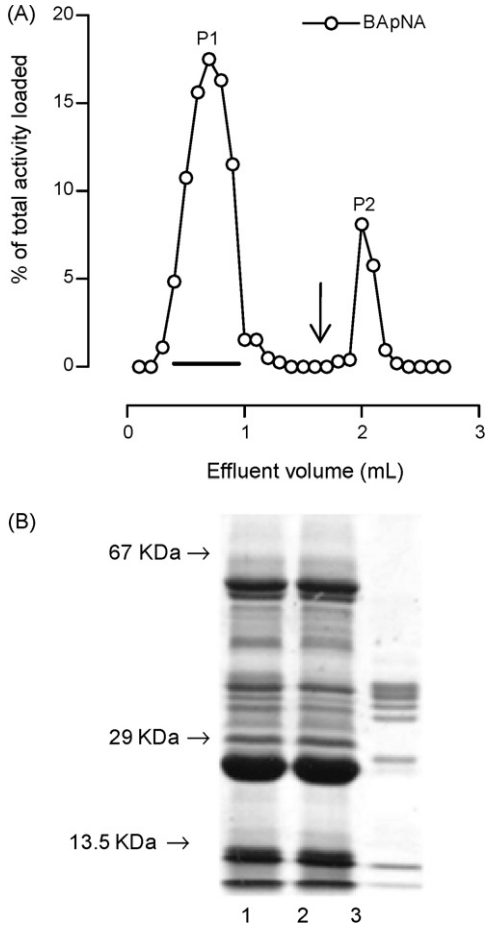

Fig. 2. Isolation of *B. moojeni* venom proteases by affinity chromatography on a column of [Arg5]-SFTI-1-agarose. (A) A solution of 5.6 mg of crude *B. moojeni* venom in 560 μL of 0.1 M Tris-buffered saline, pH 8.1, was applied to the column and thoroughly washed with buffered saline to remove unbound proteins (P1); specifically bound proteins (P2) were then eluted with 20 mM HCl (arrow). The BA*p*NA-hydrolyzing and the plasma clotting activities of the fractions are indicated. (B) Non-reducing SDS-PAGE analysis on 11% polyacrylamide gel of 70 μg of *B. moojeni* venom (lane 1), and equivalent amounts of P1 and P2, panel A (lanes 2 and 3, respectively).

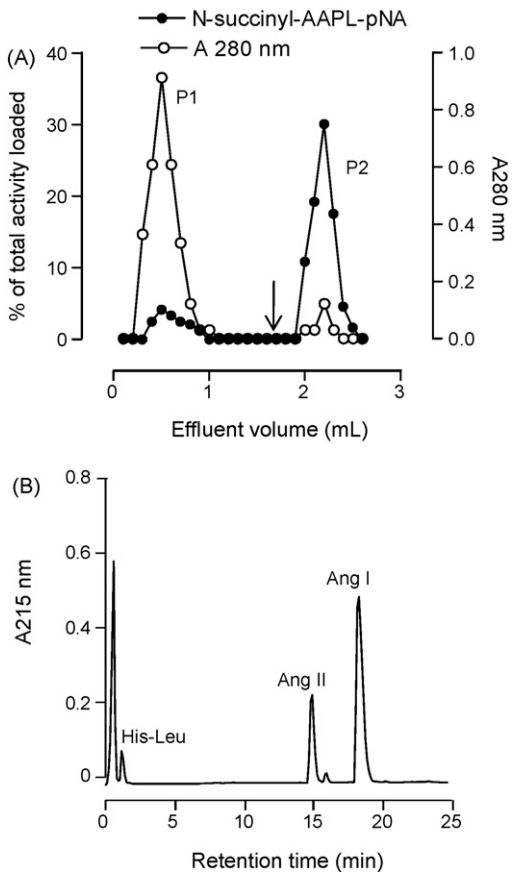

Fig. 3. Isolation of angiotensin converting enzymes from rat mesenteric arterial bed perfusate by affinity chromatography on [Leu5]-SFTI-1-agarose column. (A) A sample of 0.5 mL of 120-fold concentrated perfusate in 0.1 M Tris-buffered saline, pH 8.1, was applied to the column and chromatographed as described in Section 2.5. Proteolytic activities and protein contents of the fractions are indicated. (B) HPLC analysis of Ang I fragments generated by the affinity-purified proteases recovered in P2, panel A.

named OSFEI II [8], for instance, is a likely candidate for preparing affinity sorbent for elastases since it is an optimized inhibitor for this porcine pancreatic enzyme with sub-micromolar K_i value. In our experience, porcine elastase passed unretarded through both [Lys5]-SFTI-1-agarose and [Phe5]-SFTI-1-agarose columns (Fig. 1A and B), emphasizing the potential binding selectivity attainable with SFTI-1 analogues as ligands of affinity resins for isolation of serine proteases. Moreover, the possibility for creation of SFTI-1 analogues with novel specificities is increased by the use of non-proteinaceous amino acids [31–33] and *N*-substituted glycyl residues [7,34], in which case analogues less prone to proteolysis are usually produced; thus, preparation of SFTI-1-based affinity chromatographic resins featuring improved stability and selectivity can be achieved using simple synthetic protocols. In our experience affinity sorbents prepared with regular peptidic SFTI-1 analogues are sufficiently stable to make them useful in basic and applied biochemical research; the binding capacity performance of a single [Lys5]-SFTI-1 agarose column was evaluated over the course of six sequential chromatographic runs using saturating amounts of purified trypsin as the sample, whose repetitive yields were 15, 17, 13, 10, 5 and 5 mg of enzyme recovered per mL of packed gel. These results indicate that SFTI-1-based affinity sorbents described in this work, despite their analytical and preparative potential, should be used with the caveat of their limited stability. Although there are reports indicating that analogous affinity resins, prepared by immobilization of protein inhibitors of serine proteases, can be reused for

several experiments after due regeneration step [11], no systematic studies were carried out concerning the stability of any of these supports to allow direct comparison with the results shown for the [Lys5]-SFTI-1 agarose resin.

The acyclic SFTI-1 analogue [Arg5]-SFTI-1 has a K_i against bovine trypsin two orders of magnitude higher than that of its [Lys5]-SFTI-1 counterpart and display some inhibitory activity against bovine chymotrypsin (Table 1). Despite the apparent lack of inhibitory selectivity of the [Arg5]-SFTI-1 analogue toward serine proteases, we empirically demonstrated that [Arg5]-SFTI-1-agarose was a useful affinity support for the fractionation of the BA*p*NA-hydrolyzing enzymes of the *B. moojeni* snake venom, this a complex mixture of proteases and other proteins; thus, we found that only about 15% of the BA*p*NA-hydrolyzing activity of the venom was retained by the [Arg5]-SFTI-1-agarose resin, whereas the bulk of this activity co-eluted with the thrombin-like activity which was recovered nearly quantitatively in the breakthrough volume of the column (P1, Fig. 2A). Further analysis of the specifically bound BA*p*NA-hydrolyzing proteases recovered in P2, Fig. 2A, indicated that they were devoid of coagulating activity and showed well-defined protein bands on SDS-PAGE analysis (Fig. 2B, lane 3). Most of the proteins of this subset of venom BA*p*NA-hydrolyzing enzymes, recovered in P2, Fig. 2A, feature biochemical and enzymological properties previously described for the platelet-aggregating serine proteases of *B. moojeni* venom [35]. Thus, we believe it is now possible to isolate these venom components by affinity chromatography using [Arg5]-SFTI-1-agarose columns, profiting from the inherent advantages of this approach over more conventional procedures used thus far for the purification of these platelet-aggregating enzymes. Interestingly, affinity chromatography of *B. moojeni* venom over *p*-aminobenzamidine-agarose column at 4 °C yielded highly purified thrombin-like enzyme [36,37], a protease that passed unretarded through the [Arg5]-SFTI-1-agarose column in spite of its arginyl amidase activity (Fig. 2A); this observation stresses that peculiarities in inhibitory properties of SFTI-1 analogues can be taken to advantage for enlarging the supply of affinity sorbents for the isolation of serine proteases.

The rat mesenteric arterial bed perfusate is another example of a protease-rich biological fluid that could be successfully fractionated using an immobilized SFTI-1 analogue. The resin bearing the [Leu5]-SFTI-1 moiety, 0.6 mL packed in a 0.6 cm × 2.0 cm column, allowed that a single-step procedure was used for the isolation of the Ang II-forming elastase-2 from 60 mL of perfusate. The purified elastase-2 displayed *N*-succinyl-AAPL-*p*NA-hydrolyzing activity (P2, Fig. 3A; 42% yield) and was capable of catalyzing the Ang I to Ang II conversion reaction (Fig. 3B), as previously described for this enzyme [20], indicating that the steps involved in the affinity purification protocol were gentle enough to preserve the enzyme activity. More importantly, the elastase-2 was obtained free of other proteases, as judged by the fragments formed when 30 nmol of Ang I were incubated with an aliquot of 0.3% of the affinity-purified enzyme for 30 min at 37 °C; while the HPLC analysis of the products formed in this reaction showed only Ang II and the dipeptide His-Leu (Fig. 3B), characteristics of the Ang I to Ang II conversion reaction, several other peptides were formed when Ang I is incubated with whole perfusate, revealing the presence of other proteases such as chymases and carboxypeptidases in this fluid [38]. It is noteworthy that the [Leu5]-SFTI-1-agarose resin was capable of discriminating between different serine proteases of the perfusate, a finding that may indicate the potential of the [Leu5]-SFTI-1 and other eventual tailored synthetic SFTI-1 mutants for providing tools for revealing molecular details of the so-called alternative Ang II-forming pathways, an active area of investigation concerning tissue renin-angiotensin systems [39,40].

4. Conclusion

In conclusion, we have shown here that affinity chromatographic media exhibiting selective specificities toward particular serine proteases or groups thereof can be conveniently prepared by immobilizing different synthetic SFTI-1 analogues on agarose beads. Notwithstanding the applicability of other coupling procedures, we found that covalent attachment of SFTI-1 analogues to hydrazide–agarose via hydrazone linkage can be easily performed, leading to stable, high capacity affinity resins. If required, further stabilization of the peptide–resin linkage can be accomplished by reduction of the hydrazone function with cyanoborohydride [41]. Also, because such coupling process does not seem to impair the biological functionality of the SFTI-1 framework, any further development of SFTI-1 analogues bearing novel inhibitory specificities could be used to advantage in the preparation of affinity resins for selective isolation of the respective target proteases.

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