Identification of protease inhibitors from the human peptidome to develop novel antivirals

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Abstract

Protease inhibitors are potent modulators of the viral replication cycle by selectively interfering with host cell and viral protease functions, thus effectively preventing the propagation of infectious progeny virions. The human body produces a myriad of peptides and proteins of physiological relevance1; some play critical roles in controlling viral pathogens. Exploration of the human peptidome by body fluid analyses has revealed new therapeutic candidates against bacteria and viruses[1]. Protease inhibitors have also been found in the search for antiviral agents by bioassay-guided purification from body fluids[2,3]. However, the isolation and further analysis of low-abundance protease inhibitors can be challenging, and a different approach may often be needed to remove the impurities and concentrate the active molecules. In the present work, we used affinity chromatography and mass spectrometry to discover the main serine-protease inhibitors from a human hemofiltrate pool. Chromatographic fractionation of 1000L HF using pH-gradient cation-exchange chromatography yielded four pools by stepwise elution in increasing order of pH. A sample of each pool and the non-retained fraction were subjected to affinity chromatography on an immobilized-Trypsin gel based on a cheap and available protease used as a prototype to capture serine-protease inhibitors. Subsequently, the eluates were analyzed using a bottom-up mass spectrometry approach to sequence the peptides/proteins. Several serine-protease inhibitors were identified, with Kazal-type 1 showing the highest abundance in HF. Although much less abundant, serine-protease inhibitor Kazal-type 5 and the cysteine-protease Cystatin C3 were also found among the major inhibitors. Further analyses will reveal their antiviral activity and whether these molecules are composed of full-length proteins or fragments that can display antiviral activity. We expect this approach to be applied not only to serine-protease inhibitors but also extended to other relevant proteases for discovering novel protease inhibitors with antiviral activity.

[1]Bosso M *et al*. Exploiting the human peptidome for novel antimicrobial and anticancer agents. Bioorg Med Chem. 2018 Jun 1;26(10):2719-2726.

[2]Wettstein L *et al*. Alpha-1 antitrypsin inhibits TMPRSS2 protease activity and SARS-CoV-2 infection. Nat Commun. 2021 Mar 19;12(1):1726.

[3]Hayn M *et al*. Natural cystatin C fragments inhibit GPR15-mediated HIV and SIV infection without interfering with GPR15L signaling. Proc Natl Acad Sci USA. 2021 Jan 19;118(3).

General strategy

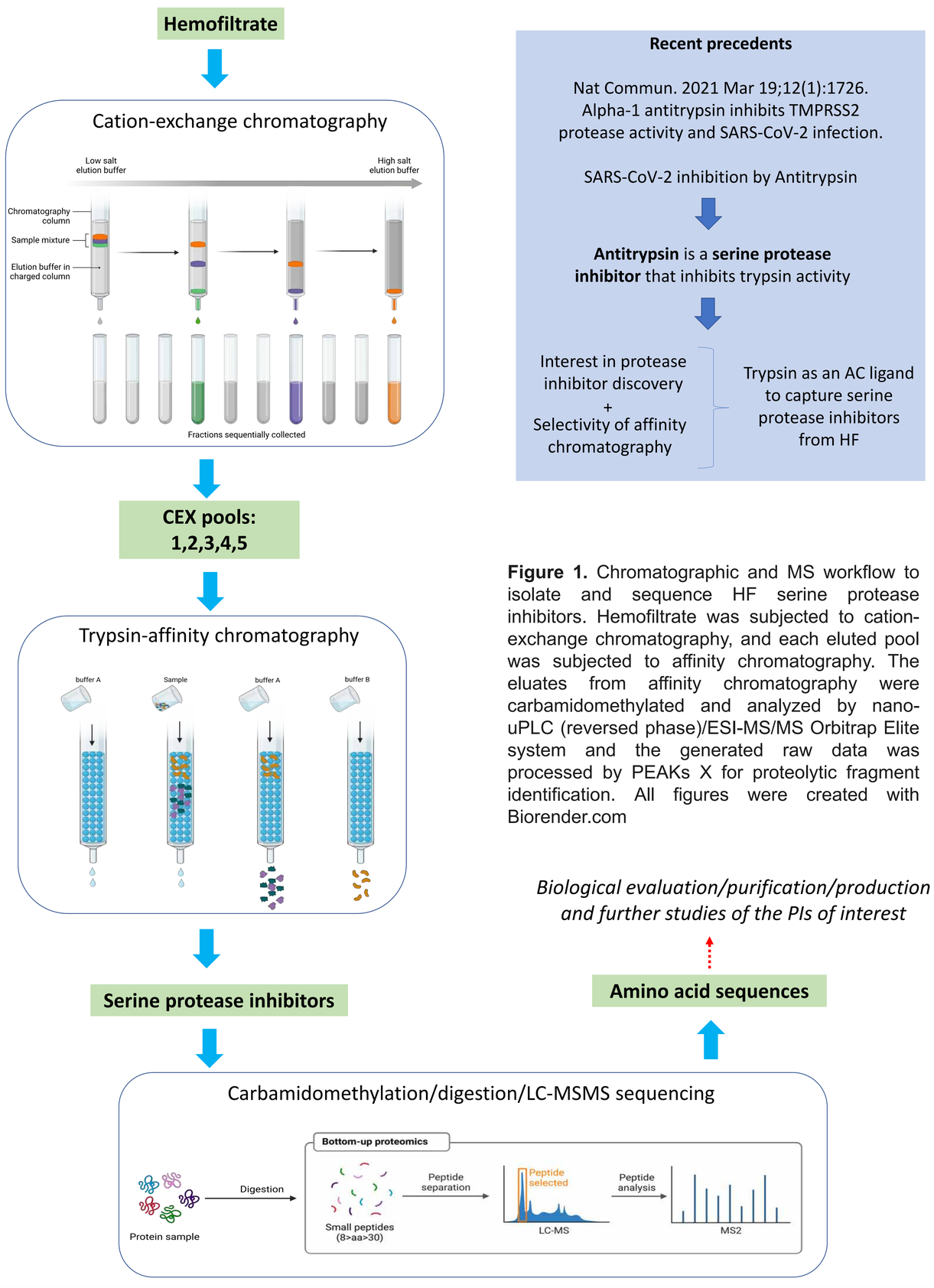

Figure 1. Chromatographic and MS workflow to isolate and sequence HF serine protease inhibitors. Hemofiltrate was subjected to cation-exchange chromatography, and each eluted pool was subjected to affinity chromatography. The eluates from affinity chromatography were carbamidomethylated and analyzed by nano-uPLC (reversed phase)/ESI-MS/MS Orbitrap Elite system and the generated raw data was processed by PEAKs X for proteolytic fragment identification. All figures were created with Biorender.com

Conclusions

- Our approach, based on trypsin affinity chromatography and mass spectrometry, allowed us to specifically capture and identify the serine protease inhibitors from the hemofiltrate, mainly represented by the serine protease inhibitor Kazal-type 1, in contrast to blood/plasma studies where alpha-1-antitrypsin is highly dominant.

What's next?

- Combination of Trypsin affinity chromatography with other purification techniques and biological activity screening.
- Additional LC-MSMS analyses to define whether these are full-length peptides or fragments.
- Screening of synthetic/recombinant protease inhibitors or their fragments for antiviral activity.

Results

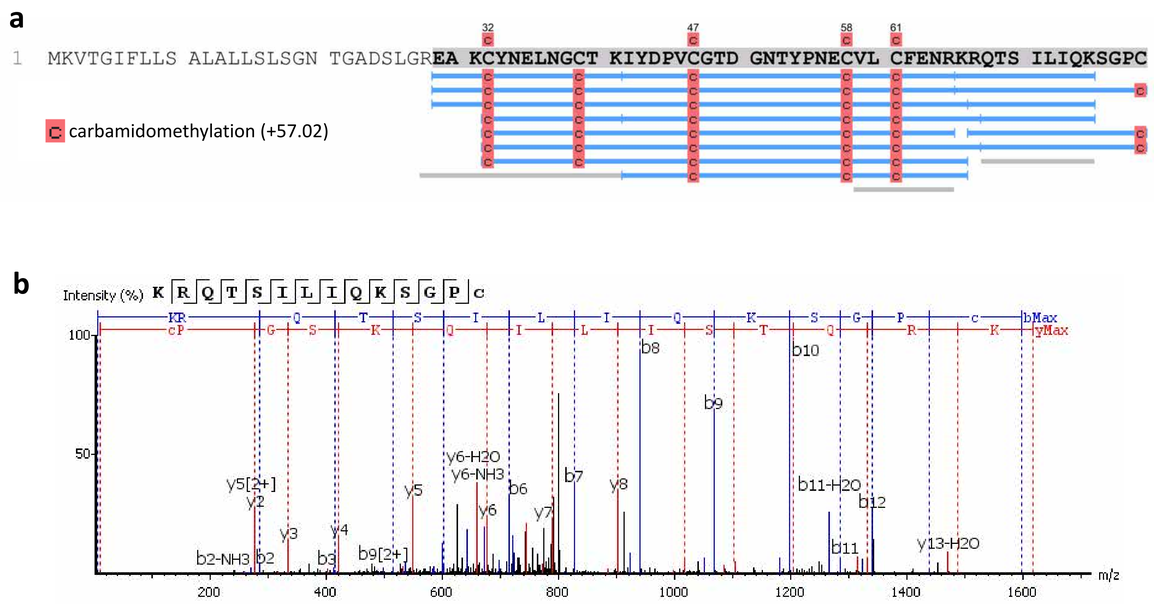

Figure 2. Mass spectrometric identification of protease inhibitors. a) Sequence coverage of the serine protease inhibitor Kazal-type 1. The blue lines represent the fragments identified by database search, while the gray lines are *de novo* peptides that were fully matched. b) MS/MS spectra of one of its peptide fragments. Both figures were generated by the proteomics software PEAKs X.

Table 1. List of protease inhibitors found in hemofiltrate by Trypsin-affinity chromatography and mass spectrometry identification

Protease inhibitor	Accession (Uniprot)	Classification
Serine protease inhibitor Kazal-type 1	P00995|ISK1_HUMAN	Serine protease inhibitor
Serine protease inhibitor Kazal-type 5	Q9NQ38|ISK5_HUMAN	Serine protease inhibitor
Cystatin-C	P01034|CYTC_HUMAN	Cysteine protease inhibitor
Kunitz-type protease inhibitor 1	O43278|SPIT1_HUMAN	Serine protease inhibitor
Antileukoproteinase	P03973|SLPI_HUMAN	Serine protease inhibitor
Tissue factor pathway inhibitor	P10646|TFPI1_HUMAN	Serine protease inhibitor
Serine protease inhibitor Kazal-type 6	Q6UWN8|ISK6_HUMAN	Serine protease inhibitor
Cystatin-M	Q15828|CYTM_HUMAN	Cysteine protease inhibitor
Alpha-1-antitrypsin	P01009|A1AT_HUMAN	Serine protease inhibitor
Cystatin-B	P04080|CYTB_HUMAN	Cysteine protease inhibitor
Kunitz-type protease inhibitor 2	O43291|SPIT2_HUMAN	Serine protease inhibitor
Elafin	P19957|ELAF_HUMAN	Serine protease inhibitor
Cystatin-SN	P01037|CYTN_HUMAN	Cysteine protease inhibitor

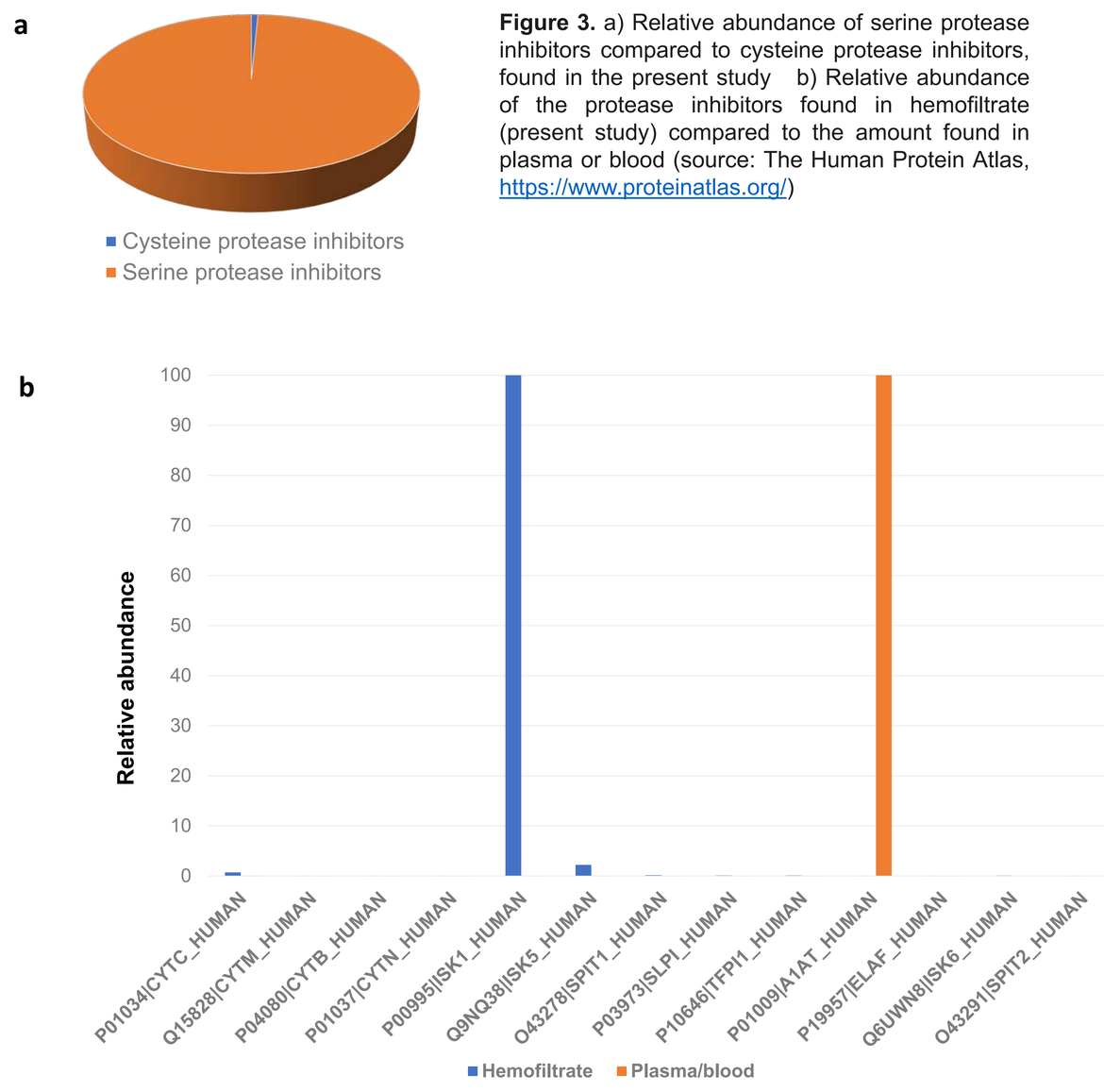

Figure 3. a) Relative abundance of serine protease inhibitors compared to cysteine protease inhibitors, found in the present study b) Relative abundance of the protease inhibitors found in hemofiltrate (present study) compared to the amount found in plasma or blood (source: The Human Protein Atlas, https://www.proteinatlas.org/)

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