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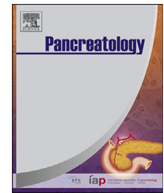
Pancreatology xxx (xxxx) xxx

Contents lists available at ScienceDirect

Pancreatology

journal homepage: www.elsevier.com/locate/pan



Substrate specificity of human chymotrypsin-like protease (CTRL) characterized by phage display-selected small-protein inhibitors

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ARTICLE INFO

Article history:
Received 4 August 2023
Accepted 15 August 2023
Available online xxx

Keywords:
Human chymotrypsin-like enzyme
Serine proteinase
Serine proteinase inhibitor
Directed protein evolution
Phage display

ABSTRACT

Chymotrypsin-like protease (CTRL) is one of the four chymotrypsin isoforms expressed in the human exocrine pancreas. Human genetic and experimental evidence indicate that chymotrypsins B1, B2, and C (CTRB1, CTRB2 and CTRC) are important not only for protein digestion but also for protecting the pancreas against pancreatitis by degrading potentially harmful trypsinogen. CTRL has not been reported to play a similar role, possibly due to its low abundance and/or different substrate specificity. To address this problem, we investigated the specificity of the substrate-binding groove of CTRL by evolving the substrate-like canonical loop of the *Schistocerca gregaria* proteinase inhibitor 2 (SGPI-2), a small-protein reversible chymotrypsin inhibitor to bind CTRL. We found that phage-associated SGPI-2 variants with strong affinity to CTRL were similar to those evolved previously against CTRB1, CTRB2 or bovine chymotrypsin A (bCTRA), indicating comparable substrate specificity. When tested as recombinant proteins, SGPI-2 variants inhibited CTRL with similar or slightly weaker affinity than bCTRA, confirming that CTRL is a typical chymotrypsin. Interestingly, an SGPI-2 variant selected with a Thr29His mutation in its reactive loop was found to inhibit CTRL strongly, but it was digested rapidly by bCTRA. Finally, CTRL was shown to degrade human anionic trypsinogen, however, at a much slower rate than CTRB2, suggesting that CTRL may not have a significant role in the pancreatic defense mechanisms against inappropriate trypsinogen activation and pancreatitis.
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1. Introduction

Chymotrypsins (CTRs) are digestive serine proteases that - by the nomenclature of Schechter and Berger [1] - preferentially hydrolyze peptide bonds after aromatic (Phe, Tyr, Trp) or aliphatic (Leu, Met) P1 hydrophobic residues that fit in the deep apolar S1 pocket of the enzyme [2]. Their inactive proenzymes (chymotrypsinogens) are produced and secreted by the exocrine pancreas and are activated in the small intestine by trypsin via limited proteolysis of the N-terminal activation peptide [3]. In human CTRs, the activation peptide remains anchored to the activated enzyme by an intramolecular disulfide-bridge. Humans express four CTR isoforms, CTRB1, CTRB2, CTRC, and a chymotrypsin-like protease, CTRL [4] [–] [6]. The latter was first identified in 1993, as one of 5 unrelated genes clustered on chromosome 16q22.111 [7], and subsequently purified and partially characterized in 1997 [6]. The protein sequence of the secreted human CTRL proenzyme is 55% identical to that of CTRB1, 56% to CTRB2, 40% to CTRC and 54% to bovine chymotrypsin A (bCTRA), a widely used model enzyme. In mice, loss of CTRL had a minimal effect on the total pancreatic chymotrypsinogen content, indicating that CTRL is a low-abundance isoform [8].

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https://doi.org/10.1016/j.pan.2023.08.004
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Please cite this article as: B.Z. Németh, Z.A. Nagy, B. Kiss *et al.*, Substrate specificity of human chymotrypsin-like protease (CTRL) characterized by phage display-selected small-protein inhibitors, Pancreatology, https://doi.org/10.1016/j.pan.2023.08.004

Besides their digestive function, chymotrypsin isoforms also play an important role in pancreatic diseases, such as chronic pancreatitis [9,10] and pancreatic cancer [11,12]. CTRC was found to protect against chronic pancreatitis by digesting potentially harmful trypsinogens and thereby promoting their degradation [9,13,14]. A similar, although biologically less significant, protective role was also demonstrated for CTRB2, and to a lesser degree for CTRB1 [10,15]. In contrast, no such function for CTRL have been reported yet. Interestingly, downregulation of *CTRL* expression in human pancreatic cancer indicated poorer prognosis, and *CTRL* was proposed as a predictive biomarker for this disease [12].

The P1 specificity of CTRL was tested on synthetic substrates revealing a clear preference for Tyr, while also accepting Phe, Trp, Leu and Met [6,16]. The *Schistocerca gregaria* proteinase inhibitor 2 (SGPI-2), is a substrate-like chymotrypsin inhibitor protein that we used previously in phage display-based directed evolution studies to decipher the specificity profile of human CTRC, CTRB1 and CTRB2 as well as the human elastases [15,17,18]. We found that *in vitro*-evolved CTRC inhibitors having Leu and Met P1 potently inhibited CTRL [17]. In the present study, we used the same phage display approach to characterize the substrate specificity of CTRL in more detail, and to complete our functional studies on human chymotrypsins.

2. Methods

Human CTRL and bovine CTRA. Recombinant zymogen human CTRL was expressed, purified and activated according to protocols published previously [16,19]. TLCK-treated bCTRA [20] was from Worthington Biochemicals (catalog number LS001432). The concentration of both enzymes was determined by active-site titration with the pan-protease inhibitor ecotin.

SGPI-2 library selection on CTRL. The SGPI-2-phage library was constructed as described previously [15]. CTRL was immobilized overnight in 12 wells of a MaxiSorp (Nunc) plate at 20 μg/mL concentration in 100 μL 100 mM Tris-HCl, 10 mM $CaCl_2$ buffer (pH 8.0). The phage selection, phage-ELISA testing, and sequencing of individual selected library members were performed as reported earlier [21]. Amino-acid sequences of clones having unique DNA sequences and at least twofold higher ELISA signal on CTRL-coated versus BSA-coated wells were used as input data for determining NNK-codon set normalized residue frequencies at the randomized positions as described. Sequence logo representation of these data was made using the WebLogo application (https://weblogo.berkeley.edu/logo.cgi, [22]).

Expression and purification of recombinant inhibitors. Wild-type recombinant ecotin was produced and purified as described previously [23]. Concentration of the ultra-pure ecotin was determined with photometry at 280 nm using its molar extinction coefficient of 23,045 $M^{-1}cm^{-1}$. Cloning, recombinant expression, and purification of the CT9 (P4-GCTYMLCR-P4′) and CT10 (P4-GCHYMLCR-P4') SGPI-2 variants were carried out as reported previously for the CT1-CT8 SGPI-2 variants [15]. The active concentration of CT9 was determined by titration against ecotin-titrated bCTRA. CT10 was titrated against ecotin-titrated CTRL.

Equilibrium inhibitory assays. Determination of the equilibrium dissociation constant (K_D) of the enzyme-inhibitor pairs was performed according to the method of the Laskowski laboratory [24], as described previously [15].

Assessing inhibitor degradation by activity assay. The CTRL and bCTRA enzymes (10 nM) were mixed with CT1, CT9 or CT10 inhibitors (11 nM) in 0.8 mL 0.1 M Tris-HCl (pH 8.0), 1 mM $CaCl_2$, 0.05% Tween 20 (final concentrations). The mixture was incubated at 25 °C. Aliquots (90 μL) were withdrawn at 0.5, 1, 2, 4, 8, 16, and 32 h and the enzyme activity was immediately measured by adding 10 μL 2 mM Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide and monitoring the release of p-nitroaniline for 3–5 min at 410 nm. In control reactions, enzymes without inhibitor were incubated and sampled identically. All enzyme-inhibitor pairs were mixed and measured twice.

Monitoring proteolytic degradation of SGPI-2 variants with HPLC and MS. The CT9 and CT10 inhibitors (15 μM) were incubated at 25 °C with bCTRA or CTRL enzymes (150 nM) in 60 mM Tris-HCl, 8.95 mM Na-HEPES, 3.43 mM $CaCl_2$, 89.5 mM NaCl buffer (pH 8.0). Aliquots were withdrawn at 0, 40, and 112 h from the CTRL mixtures, and at 0, 16, 40, 112, and 162 h from the bCTRA mixtures. The proteolytic reactions were stopped with 5% formic acid (final concentration). The samples were stored at −20 °C. Immediately before the liquid chromatography – mass spectrometry (LC-MS) measurements, the samples were incubated for 30 min at 60 °C with 5 mM TCEP (final concentration) to reduce disulfide bonds.

Samples (1 μL) were injected into a Waters Acquity I-Class UPLC system connected to a Waters Select Series Cyclic IMS (Waters Corporation, Milford, UK), hybrid quadrupole-TOF mass spectrometer. Liquid chromatographic separation of the peptides was performed on an Acquity UPLC BEH C18, 1.7 μm, 2.1 × 50 mm column. Mobile phase (A) was composed of 0.1% trifluoroacetic acid (TFA) in water, and mobile phase (B) was composed of 0.1% TFA in acetonitrile. The elution method at flow rate 300 μL/min included the following gradient: 1 min: 5% B, 25 min: 40% B, 25.5 min: 90% B at 60 °C. UV detection was performed at 220 nm using Acquity PDA detector. MS data acquisition was performed with the following parameters: 50–2000 Da, V-mode, scan time: 0.3 s, single Lock Mass: leucine-enkephalin. MS^E fragmentation was performed in the transfer cell: low energy: 6 V, high energy: ramping 25–50 V. Data were analyzed using Waters MassLynx 4.2 and spectra were inspected manually.

For peptide mapping, 1 μL of the stopped reaction samples were loaded on a Waters Acquity Premier CSH C18, 1.7 μm, 2.1 × 150 mm column for multi-step gradient elution. Mobile phase (A) was composed of 0.1% formic acid in water, and mobile phase (B) was composed of 0.1% formic acid in acetonitrile. The elution method at a flow rate of 300 μL/min included the following: 1 min: 5% B, 25 min: 40% B, 25.5 min: 90% B at 60 °C. MS data acquisition was performed with the following parameters: *m*/*z* 50–2000, V-mode, scan time: 0.3 s, single Lock Mass: leucine-enkephalin. MS^E fragmentation was performed in the transfer cell: low energy: 6 V, high energy: ramping 19–45 V. Data were analyzed using BiopharmaLynx 1.3.5 (Waters) software where MS Mass Match Tolerance and MS^E Mass Match Tolerance were set to 10 ppm and 20 ppm respectively. Digest reagent was set to non-specific and variable modification was oxidation of methionine.

Casein and trypsinogen digestion by CTRL. Bovine β-casein and human anionic trypsinogen degradation assays were carried out as described previously [15] using the indicated incubation times. We performed two independent measurements with each protein substrate.

3. Results

Directed evolution of human CTRL inhibitors from SGPI-2. Our team previously characterized the substrate-binding specificity of human CTRB1, CTRB2, CTRC, bCTRA as well as several elastase enzymes using directed evolution of the substrate-like canonical binding loop in the small-protein protease inhibitor SGPI-2 (Fig. 1) [15,17,18]. Here, we conducted the same study on CTRL. By fully randomizing the P4, P2, P1, P1′, P2′, and the P4′ positions of SGPI-2, while keeping the P3 and P3′ Cys residues intact, we generated an SGPI-2 phage library of 4.5 × 10^8 independent clones that were selected for binding to immobilized CTRL. After two panning cycles,

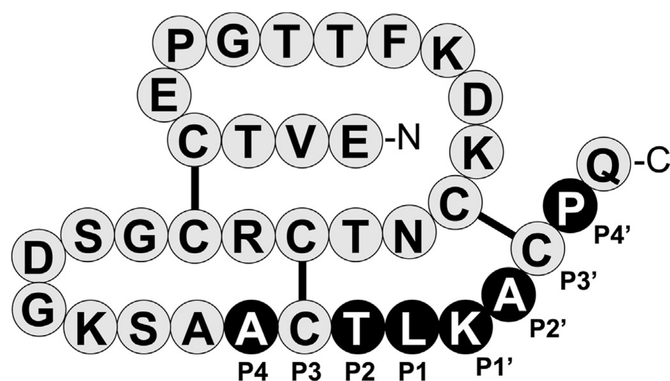

Fig. 1. Primary structure of wild-type *Schistocerca gregaria* proteinase inhibitor 2 (SGPI-2). Residues of the canonical inhibitory loop are labeled P4-P4′ according to the nomenclature of Schechter and Berger [1]. The six residues fully randomized for the library creation are highlighted with white letters and black background. Cysteine residues in disulfide bridges are connected. This figure was originally published in Ref. [17]. Copyright 2011 by The American Society for Biochemistry and Molecular Biology.

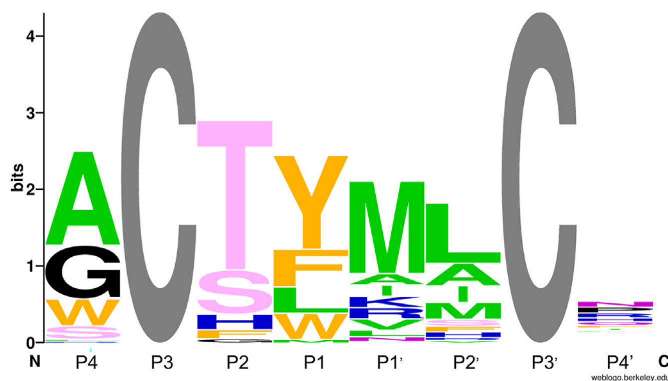

Fig. 2. Sequence logo of the P4-P4′ loop of SGPI-2 variants selected on CTRL. The single-letter amino acid code is used. Colors indicate chemical character: blue is basic, red is acidic, pink is polar with no charge, green is aliphatic, and orange is aromatic. Gly and Pro are black. The Cys residues shown in gray at positions P3 and P3′ were not randomized. The height of a column indicates the level of conservation at the position. The logo was created with the WebLogo application (https://weblogo.berkeley.edu/logo.cgi) [22].

we achieved 3600-fold enrichment of eluted clones from the CTRL-coated surface relative to the BSA-coated control. From this enriched pool, individual clones were assessed for CTRL binding in phage-ELISA, and 56 CTRL-binding clones with unique DNA sequence were identified (Table 1). The codon-normalized positional residue frequencies of these clones are represented in sequence logo format (Fig. 2). The sequence logo demonstrated a selection pattern similar to those obtained for the human CTRB and the bovine bCTRA enzymes [15], confirming that CTRL is a typical chymotrypsin.

Testing the binding-site preference of CTRL with SGPI-2 variants. In our previous study focusing on the specificity of CTRB enzymes [15], we created eight SGPI-2 variants, designated CT1-CT8. The CT1-CT5 variants have a P4-P4′ binding loop sequence of Gly-Cys-Thr-Xaa-Met-Met-Cys-Arg, differing only at the P1 position where Xaa refers to a Tyr, Trp, Phe, Leu or Met (Table 2). This set was used previously to characterize the P1 preference of the human CTRB enzymes and bovine bCTRA. The CT6-CT8 variants differ from CT1 only at their P1′ and/or P4′ positions (CT6 P1′ Lys, P4′ Arg; CT7 P1′ Met, P4′ Ala; CT8 P1′ Lys, P4′ Ala). These variants were created to test the functional contributions of positively charged residues at the P1′ and P4′ positions. To test the residue preference of CTRL at the P2′ and P2 positions, two new SGPI-2 variants were designed and produced. By a single P2′ Met to Leu replacement in CT1, we created the CT9 variant having a loop sequence of Gly-Cys-Thr-Tyr-Met-Leu-Cys-Arg, which is highly similar to the CTRL-selected consensus, Ala-Cys-Thr-Tyr-Met-Leu-Cys-Asn, the P3-P3’ segments of the two sequences being identical. Finally, by replacing the P2 Thr in CT9 with a His, which, among the few CTRL-selected, P2 hydroxyl-lacking SGPI-2 clones

Table 2
Dissociation constant (K_D) values of SGPI-2 variants on CTRL and bCTRA. The K_D values were determined after 3 h and 27 h incubation. Amino-acid differences in the binding loop of SGPI-2 variants relative to CT1 are in bold and underlined. Values are provided in picomolar unit and represent the average of two independent determinations. ND, Not determined due to the rapid degradation of the inhibitor. The bCTRA data with the CT1-CT8 inhibitors are from our previous study [15].

Inhibitor	P4-P4′ sequence	CTRL		bCTRA	
		3 h	27 h	3 h	27 h
CT1	GCTYMMCR	190	26	3.5	3.5
CT2	GCT**W**MMCR	140	140	1.8	2.7
CT3	GCT**F**MMCR	130	79	3.2	3.8
CT4	GCT**L**MMCR	190	63	3.7	3.7
CT5	GCT**M**MMCR	370	430	16	18
CT6	GCTY**K**MCR	410	58	2.5	2.1
CT7	GCTYMMC**A**	190	42	41	14
CT8	GCTY**K**MC**A**	440	130	18	6.0
CT9	GCTYM**L**CR	100	21	4.3	6.8
CT10	GC**H**YM**L**CR	520	230	ND	ND

was the most abundant residue (Table 1), we created CT10 having the binding loop sequence of Gly-Cys-His-Tyr-Met-Leu-Cys-Arg. To assess residue preference at each position, we compared the inhibitory dissociation constant (K_D) values of variants of the CT1-CT10 set. As in our previous study [15], we determined the K_D values after 3 h and 27 h incubations, to test whether affinity increases or diminishes over time, revealing a slow-binding inhibitory mechanism or proteolytic inhibitor-degradation, respectively.

***Comparative sequence logo and affinity-based analysis of the binding preference of CTRL*.** The sequence logo emerging from the CTRL-binding clones is similar to those we previously obtained for

Table 1
P4-P4′ amino acid sequence of the 56 SGPI-2-phage clones with unique DNA sequences and CTRL-binding capacity.

SCTLVLCN	GCTLAHCR	ACTWRICE	GCTLALCA	RCTLMLCA	SCTLVACR
ACHLKSCS	ACTYMRCG	ACTFRLCK	GCSLLLCY	ACTWALCP	ACTWRACR
GCTYNLCV	ACTYVMCQ	ACTFMACS	GCSYMLCP	GCSYRLCR	ACHFILCL
WCSFKACN	ACSYMLCV	WCSYKLCR	SCTWALCQ	ACTLASCI	ACSLMLCL
GCTLLLCD	GCTWMVCR	ACTYMICR	GCTYILCP	ACSYMLCR	ACTLSLCP
ACTYMLCN	SCTYMLCL	ACTLMACH	ACGFMACR	GCSFRLCN	SCSLVLCQ
ACTLARCP	ACTMMSCR	ACTYLMCF	GCTLRLCP	ACTYRLCT	
GCTYMFCI	WCSYMSCP	ACTYRLCP	ACTLMICH	ACSFMLCH	
WCTYMLCP	ACSFMLCT	LCTYMLCR	ACTYILCN	GCSFVACF	
SCSYVLCT	GCTLLLCP	GCSYMMCL	ACFLMACS	GCTWRLCR	

other chymotrypsins on the same SGPI-2 scaffold [15,17]. At the P1 position, which is typically the most important determinant of specificity, CTRL prefers, in the listed order, the same set of five residues, Tyr, Phe, Leu, Trp, Met, as other chymotrypsins, although the individual rank orders are chymotrypsin-dependent (Table 1, Fig. 2). After 27 h incubation with the CT1-CT5 inhibitors, the P1 preference order is Tyr, Leu, Phe, Trp, Met, which is almost identical with that deciphered from the sequence logo (Table 2, Fig. 3).

At the P1′ position, CTRL prefers a similar residue set as other chymotrypsins, namely Met having a long aliphatic side chain, a subset of genuine aliphatic residues, and the positively charged Lys and Arg that also have a long aliphatic segment. CTRL showed a low level P4′ preference, generally disfavoring small residues Gly (1 occurrence) and Ala (2 occurrences). Using the CT1, CT6, CT7, CT8 inhibitors, we tested the P1′ Met versus Lys and P4′ Arg versus Ala preference of CTRL and we found these to be in accordance with the sequence logo. Thus, CTRL prefers a P1′ Met over Lys by 2-3-fold, and a P4’ Arg over Ala by 2-fold (Table 2 and Fig. 4).

At the P4 and P2′ positions, CTRL selects roughly the same set of residues as CTRB1 and CTRB2, but with Ala as the most abundant choice at P4 (versus Gly in the case of CTRBs), and Leu at P2' (versus Met preferred by the CTRBs). This latter preference was also verified by the slightly higher CTRL-affinity of the CT9 inhibitor compared to CT1 (Table 2).

The only unexpected finding in the sequence logo was the relatively relaxed preference of CTRL at the P2 position, where a P2 Thr was almost exclusively selected by not only the other chymotrypsins, but several elastases and mannan-binding lectin-associated serine proteases as well [15,17,18,25]. Thr is optimal at this position as its hydroxyl forms intramolecular hydrogen bonds in SGPI-2 that stabilize the canonical loop, while its methyl group fits into the usually shallow and apolar S2 pocket of most chymotrypsin-like enzymes. Unusually, 20 out of the 56 CTRL-selected clones had a P2 residue other than Thr, and 4 of these, i.e. His (2), Phe (1) and Gly (1) lacked a hydroxyl group.

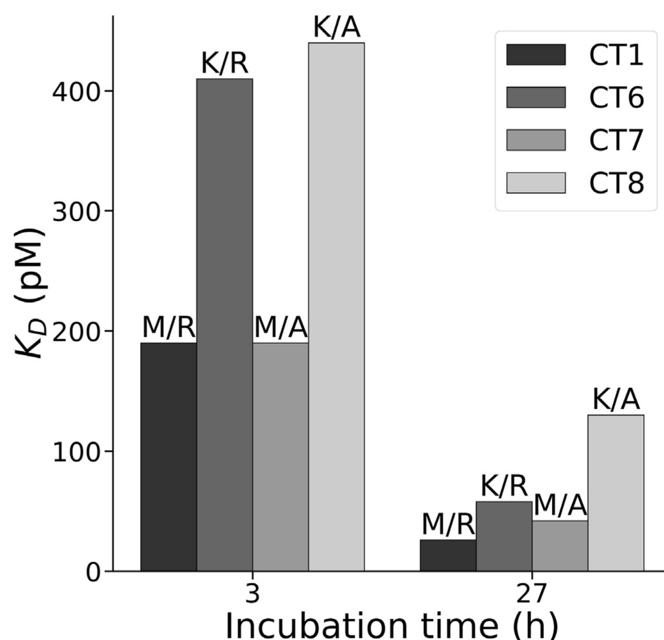

Fig. 4. Effect of P1′ and P4′ residues in SGPI-2 variants on the inhibitory efficacy against CTRL. The dissociation constant (K_D) values of the CT1, CT6, CT7, and CT8 SGPI-2 variants on CTRL were determined after 3 h and 27 h incubation. These SGPI-2 variants differ from each other only at their P1′ and/or P4′ position, indicated on the corresponding bars. CTRL has a clear preference for P1′ Met over P1′ Lys, and a slightly less distinct preference for P4′ Arg over P4′ Ala. The CTRL-inhibitory efficacy of all four inhibitors increased upon longer incubation with the enzyme.

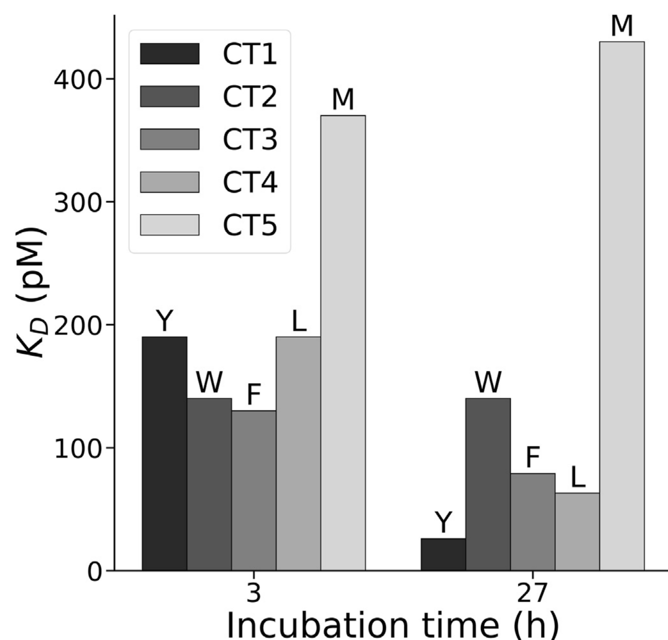

Fig. 3. Effect of P1 residue on CTRL-inhibitory efficacy of SGPI-2 variants. The dissociation constant (K_D) values of the CT1-CT5 SGPI-2 variants on CTRL were determined after 3 h and 27 h incubation. These SGPI-2 variants differ only at their P1 position, indicated on the corresponding bars. CTRL-inhibitory efficacy of CT1, CT3, and CT4 increases with incubation time, while those of CT2 and CT5 remain nearly the same. The P1 preference observed after 27 h incubation (Y, L, F, W, M, from strongest to weakest inhibition) is highly similar to the P1 preference deduced from the sequence logo.

Preferential degradation of the P2 His-containing CT10 variant by bCTRA. To determine the K_D values for CT1-CT10 on CTRL and CT9-CT10 on bCTRA (where K_D values for the CT1-CT8 set had already been published [15]), the inhibitors were first titrated with the corresponding active-site titrated enzymes. While CT1-CT8 from the previous study and CT9 from the present study were readily titrated by bCTRA, we were unable to titrate CT10 with bCTRA due to time-dependent loss of inhibitory function, suggesting proteolytic degradation. This precluded determination of a K_D for the CT10 - bCTRA pair. In contrast, we could readily titrate CT10 with CTRL and determine the K_D for the CT10 − CTRL interaction. We determined the apparent K_D values for CT1-CT10 on CTRL and CT9-CT10 on bCTRA, after 3 h and 27 h incubation (the corresponding K_D values for the CT1-CT8 set on bCTRA had already been published [15]) (Table 2). On CTRL, all but the CT2 and CT5 inhibitors showed a slow affinity maturation indicating a slow-binding type inhibition mechanism [26,27], while the K_D values for the CT2 and CT5 practically did not change indicating a simple binding mechanism. After 27 h incubation, CT10 having a P2 His inhibited CTRL 20-fold weaker than its P2 Thr mutant counterpart, CT9. In contrast, K_D values for bCTRA suggested a slow-binding mechanism for the CT7 and CT8 inhibitors carrying a P4’ Ala residue, while a simple binding mechanism was apparent for CT1-CT6 and CT9, and proteolytic degradation for CT10 (Table 2). So, while bCTRA apparently quickly degraded the P2 His-containing CT10 inhibitor, the affinity between CTRL and CT10 actually slightly increased during the extra 24 h incubation, suggesting that CTRL does not proteolytically inactivate CT10 in this time frame. We investigated this phenomenon further.

Direct evidence for proteolytic degradation of CT10* by *bCTRA. To test whether CT10 is indeed digested by bCTRA but not by CTRL,

and to demonstrate that CT1 and CT9 are not digested by these two enzymes, we mixed together bCTRA or CTRL with CT1, CT9 or CT10 with a slight inhibitor excess, and incubated them at 25 °C. At preset time points, aliquots were withdrawn, and their free enzyme concentration was assessed through measuring enzyme activity (Fig. 5A). For 5 of the 6 tested enzyme-inhibitor pairs, the level of residual enzyme activity settled to a constant level after a 1 h incubation, indicating an equilibrium state. The only exception was the bCTRA - CT10 pair, where the enzyme activity steadily increased until it reached the control level corresponding to bCTRA with no inhibitor added. Note that bCTRA is prone to autodegradation, which likely explains the observed activity loss of the bCTRA control after 4 h.

We then used RP-HPLC-MS to get direct information on the selective proteolytic degradation. We mixed bCTRA and CTRL with CT9 or CT10, incubated the mixtures at 25 °C, withdrew aliquots at various time points, and ran the samples on an RP-HPLC-MS system. With RP-HPLC we quantified, through measuring 220 nm absorbance, the change of the original intact inhibitor peak as a function of time, while the MS component verified that the peak, we monitored, belonged to the intact inhibitor. For the CTRL − CT9 and CTRL − CT10 mixtures, 3-3 samples were withdrawn at 0, 40, and 112 h, while for the bCTRA − CT9 and bCTRA − CT10 mixtures 5-5 samples were withdrawn at 0, 16, 40, 112 and 162 h (Fig. 5B). The results showed that bCTRA completely eliminated intact CT10 between 40 and 112 h, while intact CT10 started to diminish but was still detectable after 112 h in the presence of CTRL.

Unique proteolytic cleavage in CT10 suggests a specific degradation mechanism. We used LC-MS peptide-fragment analysis to determine which peptide bonds become cleaved and thereby better understand how the P2 Thr to His replacement leads to a proteolytically more sensitive inhibitor. The LC-MS peptide-fragment analysis revealed that bCTRA cleaved the P1-P1′ peptide bond of the CT10 inhibitor first, and this cleavage was followed by the hydrolysis of the peptide bond after Phe10. The Phe10 residue is structurally essential as it organizes the hydrophobic core of the SGPI-2 inhibitors [28], therefore, this second cleavage should irreversibly render the inhibitor nonfunctional. We found that the P1-P1′ peptide bond of CT9 was also cleaved by bCTRA, but importantly, this cleavage did not lead to hydrolysis of the Phe10-Lys11 peptide bond even in the 162 h sample. Importantly, in the case of the human CTRL, the P1-P1’ cleavage was detected, but occurred at a significantly slower rate, and the detrimental Phe10-Lys11 cleavage did not occur at all, thereby enabling phage-selection of SGPI-2 based CTRL inhibitors with a P2 His.

Digestion of bovine β-casein and human anionic trypsinogen by CTRL. We recently examined the proteolytic degradation of bovine β-casein and a catalytically inactive S200A mutant (S195A in bCTRA numbering) version of human anionic trypsinogen as experimental models of biologically relevant chymotrypsin

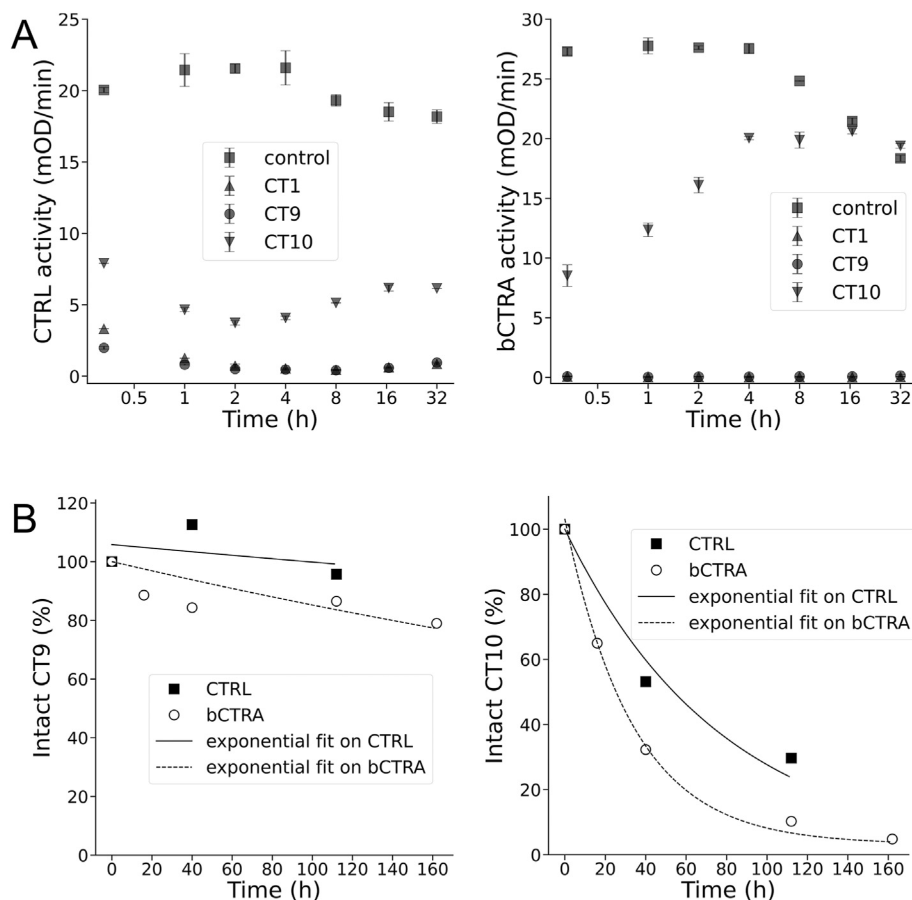

Fig. 5. Degradation of CT1, CT9 and CT10 by CTRL and bCTRA. A, Time-dependent loss of protease inhibition. Human CTRL and bovine CTRA (bCTRA) enzymes (10 nM) were incubated in the presence of (11 nM) CT1 (▲), CT9 (•), and CT10 (▼) inhibitors or with no inhibitor (■) added. The residual enzyme activity was plotted as a function of incubation time. **B, Time-dependent degradation of the CT9 and CT10 inhibitors in the presence of CTRL and bCTRA.** The inhibitor (15 μM) was incubated with 150 nM enzyme, and samples, in which the protease activity was stopped at the indicated time points, were injected into a liquid chromatography − mass spectrometry (LC-MS) system. The amount of the intact inhibitor (verified by MS) in each sample was measured as the area of the corresponding 220 nm absorbance peak in the LC chromatogram. Intact inhibitor amounts were plotted, and an exponential decay curve was fitted.

substrates [15]. In the present study, we extended these experiments to CTRL. Beta-casein and anionic trypsinogen were incubated with CTRL at 37 °C, and samples withdrawn at given times were analyzed on SDS-PAGE with Coomassie Blue staining, and quantitated by densitometry. The results showed that CTRL was capable of digesting both protein substrates (Fig. 6). The half-life of the intact casein in the presence of CTRL calculated from exponential decay curves fitted on the mean of the measured values was 8.9 min. This value is 2-fold, 2.2-fold, and 4.9-fold longer than the half-life of casein with bCTRA (4.4 min), CTRB1 (4.1 min), and CTRB2 (1.8 min), respectively, obtained with the same experimental method. The half-life of anionic trypsinogen in the presence of CTRL was 243 min, which is 3.2-fold, 1.8-fold, and 9.7-fold longer than observed with bCTRA (75 min), CTRB1 (132 min) and CTRB2 (25 min), respectively. Note that the half-lives of casein and trypsinogen cannot be compared directly, due to the different enzyme-to-substrate ratios. On both protein substrates, CTRL showed lower activities than the previously measured other chymotrypsins.

4. Discussion

In the present study, we set out to characterize the substrate specificity of human CTRL using phage-display selection, binding experiments with recombinant inhibitors, and digestion assays with bovine ß-casein and human anionic trypsinogen. By comparing our new results on CTRL to those of our previous studies on other human chymotrypsins, we found that the specificity of CTRL did not differ appreciably from those of CTRB1, CTRB2, or bCTRA [15]. The slightly different P1 preference among chymotrypsins ensures that the combined chymotrypsin set can function with high-efficiency on all protein and peptide substrates containing P1 Trp, Tyr, Phe, Leu, or Met.

The only unexpected difference from previous results was the relatively frequent occurrence of SGPI-2 clones with non-Thr/Ser P2 amino acids, His in particular, among the CTRL-selected clones. As we demonstrated by the pair of CT9 and CT10 SGPI-2 variants differing only at their P2 residue, which is Thr in CT9 and His in CT10, CTRL can be inhibited for days by both, while bCTRA is inhibited only by CT9. We found that bCTRA quickly, while CTRL slowly degrades CT10, and more importantly, unlike bCTRA, CTRL is unable to irreversibly degrade these inhibitors by hydrolyzing the Phe10-Lys11 bond. Natural, efficient reversible serine protease inhibitors interact with the enzyme through their canonical loop having an optimal substrate-like structure that therefore can be cleaved at the P1-P1′ bond. However, intramolecular interactions between the loop and the rest of the inhibitor stabilize the conformation of the cleaved binding loop resulting in a thermodynamic equilibrium of cleaved and intact inhibitors demonstrated by the fact that the cleaved forms remain functional inhibitors and the cleaved P1-P1’ bond is reversibly re-synthesized by the target enzyme [29]. In accordance with this model, our results indicate a slow accumulation of the P1-P1′ hydrolyzed form even of the strong CT9 inhibitor over long exposure to bCTRA.

For interpreting the results obtained with CT10, first we considered two models. The Thr to His replacement might generate a local conformational change exposing a certain chymotryptic cleavage site that was hidden in the parental form, or it renders the overall protein structure more flexible exposing several originally hidden cleavage sites. Based on the HPLC-MS peptide fragment analysis a third model better explains the observed results. The P2 His alters the conformation and stability of the canonical loop such that the P1-P1′ bond is more quickly hydrolyzed than in the case of a P2 Thr. Perhaps even more importantly, loss of intramolecular loop-stabilizing H-bonds in the P2 His variant hinders re-synthesis of the loop and thereby permanently exposes other proteolytic sites, such as the hydrophobic core residue Phe10, which is crucial for the stability of the SGPI-2 scaffold [28]. This way, in the P2 His variant, the P1-P1′ hydrolysis indirectly renders the inhibitor non-functional. In the 3 h phage-selection phase, CTRL successfully selected some P2 His containing clones. The HPLC-MS study revealed that upon longer incubation, CTRL is also capable of hydrolyzing the P1-P1’ peptide bond, however, for some reason that remains to be explained, CTRL cannot destabilize the structure of the inhibitor to an extent required for the detrimental cleavage of the Phe10-Lys11 bond. In all, these results support previous observations that P2 Thr is an essential structural element for SGPI-2 scaffold inhibitors.

The fact that - similarly to other human chymotrypsins [15] - CTRL digested β-casein and human anionic trypsinogen, further ascertains CTRL as a genuine chymotrypsin. Nevertheless, due to its relatively low trypsinogen-digesting activity and low abundance in the pancreas, CTRL is not expected to have a significant protective

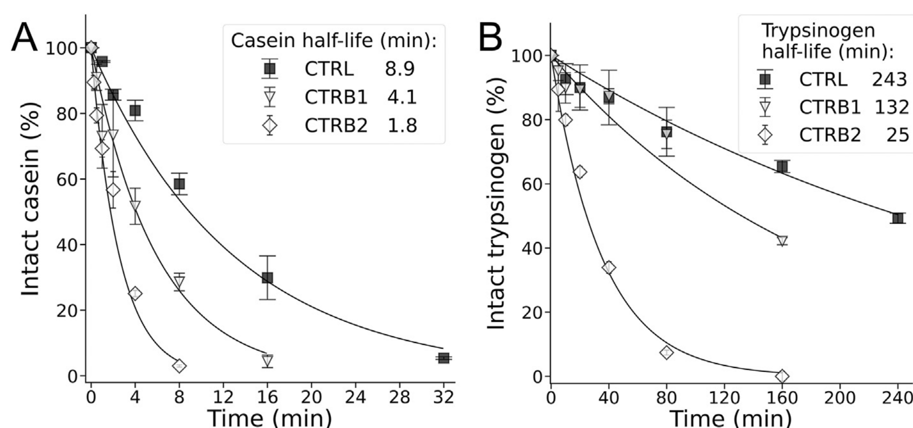

Fig. 6. Degradation of bovine β-casein and human anionic trypsinogen by CTRL. Samples were analyzed on 15% SDS-polyacrylamide gels with Coomassie Brilliant Blue R-250 staining followed by densitometry. Averages from two separate measurements were plotted, and an exponential decay curve was fitted on the mean values. For reference, results of the same assay with CTRB1 and CTRB2 from our published work [15] are also presented. Half-lives indicated in the insets were calculated from the fitted curves. **A, Casein degradation as a function of time.** Bovine β-casein (0.2 mg/mL) was incubated with 5 nM CTRL at 37 °C. At the indicated times, 100 μL aliquots were precipitated with 15 μL 100% trichloroacetic acid solution (13% final concentration) to stop the enzymatic reaction. **B, Trypsinogen degradation as a function of time.** The S200A mutant human anionic trypsinogen (1 μM) was incubated with 50 nM CTRL at 37 °C. The reaction was stopped at the indicated times by precipitation of 150 μL aliquots with 22.5 μL 100% trichloroacetic acid (13% final concentration).

role against pancreatitis. This prediction is consistent with available data from human genetic studies where an association between *CTRL* gene variants and chronic pancreatitis has not been demonstrated yet. In contrast, loss-of-function variants in CTRC have been consistently shown to increase pancreatitis risk [30–35]. Furthermore, an inversion at the *CTRB1-CTRB2* locus, which increases CTRB2 expression was found to have a protective effect against pancreatitis because CTRB2 degrades human anionic trypsinogen better than CTRB1 does [10]. We recently reported that this unique property of CTRB2 is due to the presence of the Arg236 residue, which is replaced by an Asp in CTRB1 [15].

To delineate the potential role of CTRL in pancreas health and pathology, a knockout mouse model was created [8]. Biochemical experiments with recombinant, purified mouse trypsinogens and mouse CTRL confirmed that mouse CTRL was capable of degrading cationic (isoform T7) and anionic (isoform T8) mouse trypsinogens, with cleavages after residues Tyr29 and Phe150, respectively. In *Ctrl-KO* mice, total chymotrypsin activity of pancreas homogenates was only slightly reduced relative to wild-type C57BL/6N mice, indicating that CTRL is a minor isoform. *Ctrl-KO* mice showed no overt phenotype and the pancreas was histologically normal. When intrapancreatic trypsin activation was induced by injection of a supramaximal stimulatory dose of the cholecystokinin analog cerulein, *Ctrl-KO* mice exhibited a statistically non-significant trend for higher trypsin activity, relative to wild-type mice, indicating that CTRL might contribute to the anti-trypsin defenses of the pancreas to a small extent. When acute pancreatitis was induced by repeated cerulein injections, parameters of severity in *Ctrl-KO* mice were either unchanged or showed only small increases compared to wild-type mice. Taken together, the mouse studies indicated that CTRL is a minor chymotrypsin isoform that plays no significant role in cerulein-induced pancreatitis in mice [8].

In conclusion, here we characterized the substrate specificity of human CTRL by various methods, and found that this enzyme is a typical chymotrypsin, highly similar to CTRB1, CTRB2, and bCTRA. The trypsinogen-degrading activity of human CTRL, however, was lower than those of other human chymotrypsins. The observations suggest that CTRL plays no significant role in the defense mechanisms against pancreatitis. This conclusion is in agreement with experimental pancreatitis studies using CTRL-deficient mice and available human genetic data, which showed no association between *CTRL* gene variants and pancreatitis.

Acknowledgements

This work was supported by the following grants: VEKOP-2.3.2-16-2017-00014 to GP from the European Union and the State of Hungary, co-financed by the European Regional Development Fund from the Ministry of Human Capacities in Hungary in the frame of Institutional Excellence Program for Higher Education. GP was supported by project no. 2018–1.2.1-NKP-2018-00005 that has been implemented and provided from the National Research, Development and Innovation Fund of Hungary, and financed under the 2018–1.2.1-NKP funding scheme. From the National Research Development and Innovation Office (Hungarian Scientific Research Fund), grants K119386 and K135289 were provided to GP. BZN received professional support from the doctoral student scholarship program of the co-operative doctoral program of the Ministry of Innovation and Technology financed from the National Research, Development and Innovation Fund. Contribution of GS was supported by the Lendület (Momentum) Program (LP2020-9/2021) of the Hungarian Academy of Sciences. Project no. RRF-2.3.1-21-2022-00015 has been implemented with the support provided by the European Union. This work was also supported by the National Institutes of Health (NIH) grants R01 DK082412 and R01 DK117809 to MST.

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