



Communication Rational Design of a Calcium-Independent Trypsin Variant

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Abstract: Trypsin is a long-known serine protease widely used in biochemical, analytical, biotechnological, or biocatalytic applications. The high biotechnological potential is based on its high catalytic activity, substrate specificity, and catalytic robustness in non-physiological reaction conditions. The latter is mainly due to its stable protein fold, to which six intramolecular disulfide bridges make a significant contribution. Although trypsin does not depend on cofactors, it essentially requires the binding of calcium ions to its calcium-binding site to obtain complete enzymatic activity and stability. This behavior is inevitably associated with a limitation of the enzyme's applicability. To make trypsin intrinsically calcium-independent, we removed the native calcium-binding site and replaced it with another disulfide bridge. The resulting stabilized apo-trypsin (aTn) retains full catalytic activity as proven by enzyme kinetics. Studies using Ellmann's reagent further prove that the two inserted cysteines at positions Glu70 and Glu80 are in their oxidized state, creating the desired functional disulfide bond. Furthermore, aTn is independent of calcium ions, possesses increased thermal and functional stability, and significantly reduced autolysis compared to wildtype trypsin. Finally, we confirmed our experimental data by solving the X-ray crystal structure of aTn.

Keywords: protease; trypsin; calcium-binding; enzyme engineering

1. Introduction

In nature, the serine protease trypsin (EC 3.4.21.4) is involved in the digestive hydrolysis of proteins into peptides in many vertebrates and has been used widely in various biotechnological processes. The enzyme cleaves peptide bonds on the carboxyl side of lysine and arginine. Catalytic activity is mediated by three highly conserved residues corresponding to the catalytic triad H57, D102, and S195 (chymotrypsinogen numbering) [1,2]. Like many other proteases, trypsin is synthesized as an inactive zymogen and converted into the active enzyme by limited proteolytical cleavage between Arg15 and Ile16 [3]. This cleavage induces conformational changes in the enzyme by forming a salt bridge between Ile16 and Asp194. This interaction stabilizes the substrate-binding site and the oxyanion hole, solidifying the transition state negative charge of the scissile peptide bond carbonyl oxygen [4,5]. The stability of the active state of trypsin is commonly mediated by three disulfide bonds (C42-C58, C168-C182, and C191-C220), which mainly contribute to the high catalytic activity, substrate specificity as well as catalytic robustness in non-physiological reaction conditions [6]. Furthermore, the enzyme has no need for cofactors, which is crucial for many applications.

Nevertheless, trypsin is highly dependent on Ca^{2+} -ions [7]. There are two calciumbinding sites within the enzyme. The first one is localized in the zymogenic peptide and necessary for activation processes via enterokinase. The other calcium-binding site is localized in the calcium-binding loop (CBL) and is necessary for the enzyme's activity. The absence of calcium ions leads to a considerable increase in autodigestion [8]. Furthermore, a temperature-dependent trypsin activation by calcium ions can be observed [7]. This



Citation: Simon, A.H.; Liebscher, S.; Kattner, A.; Kattner, C.; Bordusa, F. Rational Design of a Calcium-Independent Trypsin Variant. *Catalysts* **2022**, *12*, 990. https://doi.org/10.3390/ catal12090990

Academic Editors: Chia-Hung Kuo, Chun-Yung Huang, Chwen-Jen Shieh and Cheng-Di Dong

Received: 1 August 2022 Accepted: 29 August 2022 Published: 1 September 2022

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Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). Ca²⁺-induced thermal stability is also described for other proteases like thermolysin or subtilisins [9,10].

One of the most striking uses of trypsin is the detachment of adherent cells from culture flasks in animal cell cultures. Since exogenous calcium causes cell differentiation and inhibition of proliferation, trypsin is used with EDTA for this application [11]. Since trypsin activity and stability are Ca^{2+} dependent, the use of EDTA makes it necessary to increase the amount of enzyme, which could alter the physiology, protein expression, and metabolism of cultured cells [12].

Besides cell culture, trypsin is also the most used enzyme in proteomics. Its cleavage carboxyterminal of Arg and Lys results in a positive charge at the peptide C-terminus, being advantageous for MS analysis [13]. Furthermore, some MS applications would profit from a Ca²⁺-free buffer system due to the prevention of insoluble salts resulting in a possible decrease of ion signals due to buffer-induced ionization suppression [14].

The insertion of artificial cysteines forming cross-linkage within the protein of interest to increase stability is valuable in protein chemistry [15–17]. In the case of subtilisin, the deletion of the calcium-binding loop leads to a drastic stability reduction, which can be restored by inserting a disulfide bridge within the enzyme [18]. This work demonstrates that reduced stability in subtilisin can be compensated by inserting a disulfide bridge. In the present work we were able to show that in trypsin the calcium binding site can even be directly replaced by a disulfide bridge, and thus a more stable, calcium-independent trypsin variant was generated.

2. Results and Discussion

2.1. Biosynthesis and Titration of Free Thiols

In the CBL of anionic rat trypsin II (Tn), the Ca²⁺-ion is coordinated by electrostatic interactions with the side chain of Glu70 and Glu80 and the backbone carbonyl oxygens of Asn72 and Val75 (Figure 1b) [19]. The distance between C α atoms of Glu70 and Glu80 is 5.6 Å and fits ideally to the length of a disulfide bond. This fact, and a relatively high conservation score of six (determined by the bioinformatics analysis with Consurf, Figure 1a), encouraged us to create a trypsin variant with an additional disulfide bridge at this position [20]. Therefore, a calcium-independent, stabilized apo-trypsin (aTn) bearing the amino acid substitutions E70C and E80C was generated by site-directed mutagenesis, expressed as inactive zymogen in *Saccharomyces cerevisiae*, and finally purified and activated by enterokinase. To prove the concept, wildtype Tn (wt-Tn) and a single cysteine variant (mC-Tn) bearing a single E70C mutation were chosen as controls. Both enzyme variants were generated similarly to aTn.



Figure 1. Level of conservation and localization of the CBL of trypsin. (**a**) Illustration of the degree of conservation of trypsin. Highly conserved residues are shown in red and variable residues are shown in blue. (**b**) Depiction of the CBL of anionic rat trypsin II. The coordinating residues Glu70, Asn72, Val75, and Glu80 are shown in green and the stick form. Additionally, electrostatic interactions, as well as Ca²⁺, are shown in magenta. For analysis and the depiction, the pdb-file 1ITZ was used.

After biosynthesis and preparation, Tn species were investigated for the presence of free thiol functionalities, to indirectly determine the formation of disulfide bonds. Therefore, free thiols were titrated with Ellmann's reagent (DTNB: 5,5'-dithio-bis-(2-nitrobenzoic acid)), which enables detection of reduced cysteine side chains by converting DTNB to 2-nitro-5-thiobenzoate, which can be quantified spectrophotometrically (Figure S1) [21]. In the case of wt-Tn, no absorption signal at 412 nm, correlating to thionitrobenzoic acid products, was detected. This result implies that all 12 cysteines are disulfide-bridged (Table 1, Figure S2). In contrast, 65% of mC-Tn have a free sulfhydryl moiety, indicating that the artificial cysteine is not involved in forming an intramolecular disulfide bridge. It can be assumed that the remaining 35% are involved in the formation of intermolecular disulfide bonds, resulting in the formation of dimers. This assumption is also confirmed by non-reducing SDS-PAGE (Figure S3). The aTn variant shows a proportion of free cysteines of only 4.1%, indicating that almost every one of the 14 cysteine side chains is involved in disulfide bond formation. Thus, we have generated a trypsin species containing seven disulfide bridges.

Table 1. Overview of the catalytic parameters for Bz-Arg-AMC turnover and proportion of free thiol (SH_{free}) species for Tn variants.

	aTn	wt-Tn	mC-Tn
K _M (μM) ^a	56.7 ± 2.8	70.2 ± 3.7	54.9 ± 4.8
$k_{cat}~(s^{-1})$ a	0.11	0.15	0.07
$k_{cat}/K_{M} (M^{-1} s^{-1})^{a}$	1900 ± 200	2100 ± 200	1300 ± 200
SH _{free} (%) ^b	4.1	0	65

^a Kinetic parameters were determined at 30 °C in 100 mM HEPES (pH 7.8), 100 mM NaCl, and 10 mM CaCl₂ using 16 nM of Tn variants and varying Bz-Arg-AMC from 5 to 200 μ M. Hydrolysis was monitored via fluorescence (λ_{ex} =381 nm, λ_{em} =455 nm) ^b Determination of free thiols was carried out by following the procedure described by Ellman [21]. Errors represent the standard deviation of three technical replicates.

2.2. Enzymatic Activity

Enzymatic activity of wt-Tn, mC-Tn, and aTn was determined for the substrate Bz-Arg-AMC (benzoyl-arginyl-7-amido-4-methylcoumarin, Figure S4). Enzymatic cleavage of the carboxamide bond between Arg and AMC releases 7-amino-4-methylcoumarin, which results in an increased fluorescence signal ($\lambda_{ex} = 381$ nm, $\lambda_{em} = 455$ nm). According to the Michaelis-Menten-kinetics, trypsin species were characterized by analyzing the kinetic parameters of the hydrolysis, namely k_{cat}, K_M, and k_{cat}/K_M. The v/S-plots are depicted in Figure S5, and the corresponding kinetic parameters are summarized in Table 1. As a result, the presence of artificial cysteine residues in the CBL does not influence the enzymatic activity of trypsin. Furthermore, K_M and k_{cat} are in the same order of magnitude for all Tn variants. However, the K_M value for wt-Tn is slightly higher (K_M = 70 µM) than the cysteine variants mC-Tn and aTn (K_M = 55-57 µM). On the other hand, the k_{cat} of wt-Tn is marginally higher (k_{cat} = 0.15 s⁻¹) than for aTn (k_{cat} = 0.11 s⁻¹). aTn also shows a slightly higher k_{cat} value than the single cysteine variant mC-Tn (k_{cat} = 0.07 s⁻¹). In general, all kinetic constants determined in this work are in the same order of magnitude as described in the literature for bovine trypsin (Tn(bov)) and the aforementioned Bz-Arg-AMC substrate [22].

Determination of Ca²⁺-dependency on the activity and stability of wt-Tn and aTn was carried out after 16 h incubation in the presence and absence of Ca²⁺ and subsequent activity measurements (Figure 2a). Reaction mixtures without Ca²⁺ were additionally treated with EDTA to remove remaining calcium ions by chelation. The incubation in the presence of Ca²⁺ leads to an expectable reduction of activity (A_{rel, wt-Tn} = 80%; A_{rel, aTn} = 81%), resulting from autolytic processes. On the contrary, in the absence of Ca²⁺ (which was supported by the addition of EDTA) the activity of aTn (A_{rel} = 81%) is nearly unchanged (Figure 2a). At the same time, the activity of wt-Tn is drastically reduced by 41%. This effect is explained by an increased autolysis rate due to the missing Ca²⁺ in the case of wt-Tn, indicating that aTn benefits from the artificial disulfide bond [23]. In addition, the disulfide

bridge also contributes to increased thermal stability. Corresponding measurements were carried out with the appropriate trypsinogen variants and at a lowered pH to prevent autolytic events, which would result in fragmentation of the trypsins during thermal denaturation. Thermal unfolding and refolding of wt-Tn and aTn was measured by real-time simultaneous monitoring of internal tryptophane fluorescence at 330 nm and 350 nm differential scanning fluorimetry. Melting curves are depicted in Figure 2b. Melting temperature increases from 72.4 °C in the case of wt-Tn to 81.7 °C in the case of aTn in the presence of Ca²⁺. A similar stabilizing effect is also observed in the presence of EDTA (wt-Tn = 71.6 °C, aTn = 81.1 °C). Furthermore, it was found that the aTn does not seem to denature completely even at 90 °C. Therefore, refolding of aTn is detectable, which is not the case with wt-Tn.



Figure 2. Evaluation of the contribution of the E70/80C disulfide bridge in aTn to the calcium dependence of enzymatic activity and the thermal stability (**a**) The bar chart shows the influence on the reduction of the relative activity A_{rel} in the presence and absence (EDTA) of Ca^{2+} after 16 h in comparison to the starting activity at 0 h (t₀). The activities were related to the respective starting point (A) of either aTn (shown in black) or wt-Tn (shown in blue). (**b**) The micro-thermophoresis method depicts the thermal denaturation of aTn (shown in black) and wt-Tn (shown in blue). A_{rel} was determined at 30 °C in 100 mM HEPES (pH 7.8), 100 mM NaCl, and either 10 mM CaCl₂ or 0.2 mM EDTA using 16 nM of Tn variants and 200 μ M Bz-Arg-AMC. Unfolding and folding was monitored using 20 μ M trypsin, 10 mM CaCl₂ or 0.2 mM EDTA in 20 mM MES, 150 mM NaCl, pH 5.5. The temperature was increased from 40 to 90 °C at a ramp rate of 1 °C/min. Errors represent the standard deviation of three technical replicates.

2.3. X-ray Structure Analysis

Anionic rat trypsin sometimes can be challenging to crystallize. One reason is the high proteolytic activity of the enzyme, which leads to autolysis and thus fragmentation [23]. In addition, rat trypsin variants are sometimes characterized by poor crystallizability. Therefore, as in previous studies, the sequence-like (sequence similarity 86%, for more detail see Figure S6) and structurally identical bovine trypsin was used for crystallization [24]. In addition, all variants were produced as inactive S195A variants to prevent autolysis as well as optional folding influences by inhibitors. As a positive side effect, this strategy allowed us to investigate the transferability of the motif between different trypsin species.

After biosynthesis, purification, and refolding of bovine trypsin variants, circular dichroism spectroscopy was done to prove correct protein folding, as activity measurements with the S195A mutation were not possible (Figure S7). In addition, thermodynamic denaturation was examined, showing a 4.2 °C increase in stability comparing wt-Tn(bov) S195A (67.6 °C) to aTn(bov) S195A (71.8 °C) (Figure S8). This result is similar to the stabilization effects of rat trypsin variants, although not quite as pronounced.

As a result of crystallization, the structure of aTn(bov) S195A was solved. The resolution was 1.40 Å, with a completeness of the structure of 98%. Residual 2% correspond to the region from residue 73 to 79, which could not be resolved (Figures 2 and S9). This region corresponds to the original CBL. The increased flexibility can be attributed to the missing Ca²⁺-induced coordination of the residues Asn72 and Val75. Despite this result, there is still increased structural integrity created by a new formed disulfide bridge between Cys70

and Cys80, proven by the electron density map (FoFc and 2FoFc) (Figure 3b). The correct positioning of the disulfide bond is also verified in the overlay structure of aTn(bov) S195A and wt-Tn(bov) (Figure S10).



Figure 3. Crystal structure of aTn(bov) S195A. (**a**) The whole structure is shown as cartoon. For better orientation, the catalytic center consisting of His57, Asp102, and Ala195 are marked in the green sticks. The unresolved residues 73 to 79 are shown as dashed lines. (**b**) Depicted is the introduced disulfide bridge. Both cysteine residues are shown as green sticks. In addition, the electron density (FoFc and the 2FoFc) maps are indicated as black mesh (pdb-ID 8ADT).

2.4. Conclusions

Thus, the crystal structure supports the presence of the expected additional disulfide bridge in trypsin and confirms the activity and stability measurements. Furthermore, we demonstrated that a disulfide bridge between residue 70 and 80 is formed in both rat and bovine trypsin. In both cases, this is beneficial for the stability and Ca^{2+} independence of the enzyme. This feature emphasizes a potential universal stabilization strategy for the conformation and catalytic activity of Ca^{2+} -dependent trypsins in numerous applications such as mass spectrometry and biocatalysis.

3. Materials and Methods

3.1. Construction, Biosynthesis, and Purification of wt-Trypsin and Trypsin Variants

All trypsin variants were generated by site-directed mutagenesis using Pfu DNA polymerase (Thermo Scientific, Waltham, MA, USA) and either a pST-vector (Tn) or a pET-vector (Tn(bov)) as described previously [25,26]. All mutations were introduced using pairs of complementary primers (Table S1). The sequence of all generated constructs was confirmed by DNA sequencing (LGC Genomics, Berlin, Germany). The gene constructs of Tn-variants were subsequently cloned into pYT-expression-vector and transformed into Saccharomyces cerevisiae DLM 101a cells. Biosynthesis, purification of zymogenic Tn, and subsequent activation using enterokinase (Roche Diagnostics, Mannheim, Germany) were done as described before [25]. For preventing autolytic processes, Tn variants were stored in 10 mM HCl at -20 °C. For crystallization, Tn(bov) variants were expressed as catalytic inactive species (S195A). Therefore, the respective pET-vectors were transformed into Escherichia coli BL21 (DE3) cells. After accumulating the Tn(bov) variants as inclusion bodies, isolation, refolding, purification, and subsequent activation using enterokinase were performed using previously established procedures [27]. The overall yield of Tn variants was 1.5 to 2.1 mg/l_{culture}, while the overall yield of Tn(bov) variants was 0.8 to 2.3 mg/l_{culture}. SDS-PAGE and mass spectrometry confirmed the purity and identity of all protein variants (Figure S11).

3.2. Activity Measurement of Tn

The activity of Tn and Tn variants was determined while monitoring the hydrolysis of benzoyl-L-arginine-7-amido-4-methyl coumarin (Bz-Arg-AMC, Bachem, Bubendorf,

Switzerland) at an F-310 fluorescence spectrometer (Hitachi, Tokio, Japan) [28]. In detail, 16 nM of the corresponding Tn was dissolved in 100 mM HEPES (pH 7.8), 10 mM CaCl₂, 100 mM NaCl, and 5-200 μ M Bz-Arg-AMC (dissolved in *N*,*N*-dimethylformamide). Increasing fluorescence was monitored for 5 min at 20 °C using an excitation wavelength of 381 nm and an emission wavelength of 455 nm. The catalytic properties k_{cat} and K_M were determined with the Michealis-Menten-regression from the v/[S]-regression curves (Figure S5) using Origin8.1 (OriginLab Corporation, Northampton, MA, USA) [29]. To determine Ca²⁺-dependency, wt-Tn and aTn were incubated in 100 mM HEPES (pH 7.8), 100 mM NaCl containing either 10 mM CaCl₂ or 10 mM EDTA. After 16 h of incubation, measurements using 16 nM of the corresponding Tn and 200 μ M of Bz-Arg-AMC for 5 min at 20 °C were performed.

3.3. Determination of Free Sulfhydryl Groups

Ellman's protocol was used to determine the number of accessible sulfhydryl functionalities within all protein variants to verify the correct formation of the disulfide bonds [21,30]. For calibration, a concentration of cysteamine (5–50 μ M) was used (Figure S2a). For measurement, 65 μ M of the particular trypsin variant was dissolved in 100 mM Tris/HCl (pH 7.5), and 0.1 mM of DTNB (dissolved in dimethyl sulfoxide) was added. After incubation for 5 min at room temperature, absorbance was measured at 412 nm with a NOVOstar plate reader (BMGLabtech, Ortenberg, Germany).

3.4. Determination of Thermostability

Real-time simultaneous monitoring of the internal tryptophane fluorescence at 330 nm and 350 nm during thermal unfolding and refolding of wt-Tn and aTn was measured on a Prometheus NT.48 instrument (Nanotemper, Munich, Germany) with an excitation wavelength of 280 nm [31]. Capillaries were filled with 10 μ L of a suspension containing 20 μ M of trypsin in presence of CaCl₂ (c = 10 mM) and EDTA (c = 0.2 mM), respectively (in 20 mM MES, 150 mM NaCl pH 5.5). The temperature was increased from 40 to 90 °C at a ramp rate of 1 °C/min, with one fluorescence measurement per 0.027 °C. The ratio of the recorded emission intensities (Em_{350nm}/Em_{330nm}) was plotted as a function of the temperature. The fluorescence intensity ratio and first derivative were calculated with the manufacturer's software (PR.ThermControl).

3.5. Crystallization

The protein solution of aTn(bov) S195A was concentrated to a final concentration of 10 mg/mL in 50 mM HEPES/NaOH (pH 7.8), 100 mM NaCl, and 10 mM CaCl₂, and crystallized by hanging drop vapor diffusion at 20 °C. Equal amounts of the protein solution and precipitant solution (0.2 M KNO₃, pH 6.5, 22% (w/v) PEG 3350) were mixed and incubated at 20 °C. After approximately 14 days, the growth of trigonal crystal was observable.

Diffraction images of a single aTn(bov) S195A crystal were collected using a copper rotating-anode source (Cu K_{α} radiation (λ = 1.5418 Å), RA Micromax 007, Rigaku Europe, Neu-Isenburg, Germany) and a CCD detector (Saturn 944+, Rigaku Europe, Neu-Isenburg, Germany). Oscillation images were integrated, merged, and scaled using XDS to a resolution of 1.439 Å (for detailed information, see Table S2) [32]. All datasets were processed with the HKL2000 suite, and structures were solved using Phaser's molecular replacement method using PDB coordinate file 1MTS as a search model [33–35]. Coot and REFMAC5 were used for model building and refinement, respectively [36,37]. PROCHECK analyzed structure quality [38]. All molecular images were generated by Pymol (Schrödinger, New York, NY, USA). **Supplementary Materials:** The following supporting information can be downloaded https://www. mdpi.com/article/10.3390/catal12090990/s1. Figure S1: Determination of free sulfhydryl groups, Figure S2: Calibration curve and results for the determination of free thiols, Figure S3: Non-reducing SDS-PAGE of the Tn variants, Figure S4: Hydrolysis of Bz-Arg-AMC, Figure S5: v/[S]-regression curves of Bz-Arg-AMC conversion catalyzed by diverse trypsin variants, Figure S6: Alignment of the protein sequences of anionic rat trypsin II and cationic bovine trypsin, Figure S7: Circular dichroism (CD) measurements of bovine trypsin species, Figure S8: Thermal denaturation of aTn(bov) S195A and wt-Tn(bov) S195A measured by CD-spectroscopy, Figure S9: Overall structure of aTn(bov) S195A, Figure S10: Ca²⁺-binding site of trypsin, Figure S11: SDS-PAGE and mass spectrometry of trypsin variants, Table S1: Sequences of the primers used for site-directed mutagenesis, Table S2: Overview of the crystallization data and refinement statistics of aTn(bov) S195A [21,23,27,28,30,35,39–44].

Author Contributions: Conceptualization, S.L. and A.H.S.; methodology, A.H.S., S.L., A.H.S. and C.K.; software, C.K. and A.H.S.; validation, A.H.S., S.L. and A.K.; data curation, S.L., A.K., A.H.S. and C.K.; writing—original draft preparation, A.H.S. and S.L.; writing—review and editing, S.L., A.H.S., A.H.S., A.K. and F.B.; supervision, S.L. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the "Aninstitut für Technische Biochemie" at the Martin Luther University Halle-Wittenberg.

Data Availability Statement: Not applicable.

Acknowledgments: We thank Tobias Aumüller for the fruitful discussion. Furthermore, we thank Franziska Seifert for the opportunity to use the Nanotemper instrument and instruction on the device. In addition, we thank Milton T. Stubbs for the opportunity to use his crystallization facility. We acknowledge the financial support of the Open Access Publication Fund of the Martin-Luther-University Halle-Wittenberg.

Conflicts of Interest: The authors declare no conflict of interest.

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