

# Expression of rat chymotrypsinogen in yeast: a study on the structural and functional significance of the chymotrypsinogen propeptide

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**Abstract** The role of the propeptide sequence and a disulfide bridge between sites 1 and 122 in chymotrypsin has been examined by comparing enzyme activities of wild-type and mutant enzymes. The kinetic constants of mutants devoid of the Cys1–Cys122 disulfide-linked propeptide show that this linkage is not important either for activity or substrate specificity. However this linkage appears to be the major factor in keeping the zymogen stable against non-specific activation. A comparison of zymogen stabilities showed that the trypsinogen propeptide is ten times more effective than the chymotrypsinogen propeptide in preventing non-specific zymogen activation during heterologous expression and secretion from yeast. This feature can also be transferred in trans to chymotrypsinogen; i.e. the chymotrypsin trypsin propeptide chimera forms a stable zymogen.

**Key words:** Zymogen activation; Chymotrypsin; Disulfide bond; Protein folding

## 1. Introduction

Chymotrypsin and trypsin, the two pancreatic serine proteases in the intestine, are closely related both structurally and functionally. The vertebrate trypsins and chymotrypsins are 45% identical in their amino acid sequence, and their tertiary structures are also very similar [1]. However, the propeptide segment of zymogens is not conserved in either amino acid sequence or structure.

Besides length there are other characteristic differences between the propeptides: The shorter trypsinogen propeptide has a distinctive structure characterized by four consecutive aspartate residues directly preceding the Lys15–Ile16 peptide bond that is cleaved during activation [2,3]. The trypsinogen propeptide is mobile, and projects into the solvent [4–6], while the chymotrypsinogen propeptide is covalently linked to the Ser109–Ala132 loop via the Cys1–Cys122 disulfide bridge and several H-bonding interactions [7–9]. Due to this disulfide bond, the chymotrypsinogen propeptide remains bound to the enzyme after activation. In contrast, the trypsinogen propeptide dissociates from the enzyme.

The characteristic four aspartic acids in the trypsinogen propeptide are a crucial component of the (Asp)4Lys recogni-

tion sequence of enterokinase, the specific enzyme that activates trypsinogen. In contrast, chymotrypsinogen is activated by trypsin.

The significance of the chymotrypsin(ogen)-specific Cys1–Cys122 disulfide, if any, is unknown. The Cys1–Cys122 disulfide could link the propeptide to the protein after activation perhaps because it displays a unique function in enzyme activity and/or specificity. Alternatively it may have a role in zymogen folding. To test these possibilities we constructed mutants in which the disulfide bond was eliminated. We also examined the relative abilities of the trypsinogen and chymotrypsinogen propeptides in preventing non-specific activation of the zymogens chimera in a yeast expression system by comparing the yields and stabilities of wild-type zymogens and a chymotrypsin trypsin propeptide chimera.

## 2. Materials and methods

### 2.1. Materials

Restriction endonucleases, T4 polymerase and T4 ligase were from New England Biolabs or Boehringer. TSK Toyopearl SP-650M was bought from Supelco. High purity enterokinase was purchased from Biozyme. TPCK treated bovine trypsin for chymotrypsinogen activation, SBTI Sepharose, MUGB, MUTMAC, succinyl-AlaAlaProPhe-pNA, 1-Tyr-pNA and Bz-1-Lys-pNA amide substrates were the products of Sigma Chemical Co. The tetrapeptide amide substrates, succinyl-AlaAlaPro-Xaa-AMC were synthesized as described previously [10].

### 2.2. Cloning, mutagenesis and expression

Rat enzymes were used throughout this study (type II for trypsin). The cloning of rat trypsinogen was described previously [11]. The cloning of rat chymotrypsinogen was performed as follows. A lambda gt11 cDNA library of the rat cell line, AR425 was amplified by polymerase chain reaction using two oligonucleotides corresponding to the 5' and 3' ends of the chymotrypsinogen coding region. The oligonucleotides used, 5'GCCACCAAGCTTGTGGAGTCCCT3' and 5'GCGAGCTCAGGTGTCTTCCAAGAT3' added a *Hind*III site to the 5' end and a *Sac*I site to the 3' end.

The chymotrypsinogen mutants were constructed according to the Kunkel method [12] using oligonucleotide 5'GGCCAGTGCCAAGCTTCTGGAGTCCCT3' for the Cys1Ser substitution and oligonucleotide 5'ACTGTGTCTGCCGTAT/GCTCTACCCAACGTCGAT3' for the Cys122Ala/Ser substitution. The mutations were verified by DNA sequencing [13]. The enzymes were expressed in the yeast system described for carboxypeptidase [14] as  $\alpha$ -factor leader peptide zymogen fusion proteins. (The  $\alpha$ -factor leader peptide directs the protein into the culture medium and it is cleaved during the export.) The 5'CTCTCGACAAACGGATCGTCAACGGAGAGGATCCTATTCT3' and 5'GGCAA-TAGCATCCTCTCCGTTGACGATCCGTTTGTCTGAGAGGTAC3' oligonucleotides were used to form an  $\alpha$ -factor leader peptide-chymotrypsinogen fusion protein by linking an *Xma*I site, created in chymotrypsinogen at the Pro24 and Gly25 triplets, to the *Kpn*I site in the  $\alpha$ -factor leader peptide. For another fusion construction oligonucleotides 5'AGCTTTCCCGGTGGATGATGATAAGA-TCGTCAACGGAGAGGATGCTATTCT3' and 5'CCGGGAATAG-

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**Abbreviations:** TPCK, tosyl-L-alanyl chloromethyl ketone; SBTI, soybean trypsin inhibitor; pNA, *p*-nitroanilide; AMC, 7-amino-4-methylcoumarin; MUGB, 4-methylumbelliferyl *p*-guanidinobenzoate; MUTMAC, 4-methylumbelliferyl *p*-(*N,N,N*-trimethyl ammonium) cinamate chloride.

CATCCTCTCCGTTGACGATCTTATCATCATCATCCACCGGG-AA3' were used to link the *Xma*I site in chymotrypsin to the *Hind*III site at the junction of  $\alpha$ -factor leader peptide and trypsinogen propeptide. This construct encoded a chymotrypsin trypsin propeptide chimera that is fused to the  $\alpha$ -factor leader peptide and contains a Cys122Ser substitution.

The level of protein expression was checked by SDS polyacrylamide gel electrophoresis performed in the absence of reducing agents. 500  $\mu$ l supernatant of cultures of similar cell density were precipitated for 30 min on ice by the addition of 1 ml of absolute ethanol. The precipitates were sedimented for 15 min, dried in vacuo and resuspended in 50  $\mu$ l sample buffer. After heating on 100°C for 3 min, 15  $\mu$ l samples were loaded onto 12.5% polyacrylamide gels.

### 2.3. Enzyme purification

When the yeast culture media contained zymogens, they were isolated in inactive form on a TSK Toyopearl SP650-M cation exchanger [15], then they were activated at 37°C with an overnight enterokinase treatment (at 20 unit enterokinase/1.0 mg zymogen ratio) or with a six-hour TPCK bovine trypsin treatment (at a 2  $\mu$ g trypsin/1.0 mg zymogen ratio). After activation the activator trypsin was removed from the solution of the activated chymotrypsin (mutant) on a Benzamidine-Sepharose column. The active forms were purified by SBTI Sepharose affinity chromatography. In cases when the active forms of the enzymes rather than the zymogens were present in the culture media, they were purified by directly subjecting the cation exchange chromatography fractions to an SBTI Sepharose affinity column. The enzyme concentrations of trypsin and chymotrypsin were determined with active site titration [16] by using active site titrants MUGB and MUTMAC, respectively.

### 2.4. Enzyme assays

The enzyme activities were determined on amide substrates at 37°C with photometry (*p*-nitroanilide liberation followed by the absorbancy at 410 nm) and fluorimetry (7-amino-4-methylcoumarin liberation, followed by the emission at 460 nm with excitation at 380 nm) in a 50 mM HEPES, 10 mM CaCl<sub>2</sub>, 0.1 M NaCl buffer (pH 8.0). The data were analyzed with the KinetAsyst software. For the determination of the active enzyme concentration in the culture medium, 100  $\mu$ l aliquots of appropriately diluted culture medium were added to 70  $\mu$ l of 500 mM HEPES, 100 mM CaCl<sub>2</sub>, 1.0 M NaCl buffer (pH 8.0), 520  $\mu$ l water and 10  $\mu$ l of a 10 mM succinyl-AlaAlaProPhe-pNA (chymotrypsin substrate) or Bz-I-Lys-pNA (trypsin substrate) solution. The hydrolysis rates were measured on 410 nm. The total enzyme concentrations were determined in the same way after the activation of the zymogen forms. To this end 300  $\mu$ l aliquots of the culture medium were treated with 1.0  $\mu$ g TPCK bovine trypsin or 10.0 units enterokinase. Five minutes at 37°C for trypsinogen activation and 10 minutes on ice for chymotrypsinogen activation were sufficient to achieve maximal rates of substrate hydrolysis.

## 3. Results

To explore the structural and functional significance of disulfide bond Cys1–Cys122 present in the chymotrypsin/ogen but not in trypsin/ogen structure (Fig. 1), chymotrypsinogen mutants Cys1Ser–Cys122Ala and Cys1Ser–Cys122Ser, wild-type chymotrypsinogen, the chymotrypsin trypsin propeptide chimera chymotrypsinogen and wild-type trypsinogen were expressed in yeast. The resulting culture media were compared by SDS polyacrylamide gel electrophoresis under non-reducing conditions (Fig. 2), and by measuring their enzyme activities before and after activation of the zymogens produced (Table 1). The gel patterns of the yeast culture media indicated a high expression level for the chymotrypsin trypsin propeptide chimera chymotrypsinogen and wild-type trypsinogen (lanes 5 and 6 in Fig. 2), while the expression of the other three protein constructs, especially those of the two chymotrypsinogen mutants without disulfide bridge Cys1–Cys122 (lanes 2–3 in Fig. 2) appeared to be relatively low. Concerning the electrophoretic

|                        | 1              | 10         |
|------------------------|----------------|------------|
| Cow chymotrypsinogen A | CGVPAIQPVLSGLS | <b>Riv</b> |
| Cow chymotrypsinogen B | CGVPAIQPVLSGLA | <b>Riv</b> |
| Dog chymotrypsinogen   | CGVPAIQPVLSGLS | <b>Riv</b> |
| Rat chymotrypsinogen   | CGVPTIQPVLTGLS | <b>Riv</b> |
| Rat trypsinogen II     | FPVDDDD        | <b>Kiv</b> |
| Cow trypsinogen        | VDDDD          | <b>Kiv</b> |
| Goat trypsinogen       | CDDDD          | <b>Kiv</b> |
| Sheep trypsinogen      | FPVDDDD        | <b>Kiv</b> |
| Pig trypsinogen        | FPTDDDD        | <b>Kiv</b> |
| Horse trypsinogen      | SSTDDDD        | <b>Kiv</b> |
| Dogfish trypsinogen    | APDDDD         | <b>Kiv</b> |

Fig. 1. Trypsinogen and chymotrypsinogen N-terminal sequences. The propeptide sequences are in capital letters, and the amino acids are bold between which the peptide bond is cleaved during activation.

patterns it has to be reminded that electrophoretic mobilities of proteins in the SDS gel under non-reducing conditions are not only determined by their molecular weight but also by the actual compactness and shape of the proteins. This may explain, for example, why wild-type trypsinogen with six interchain disulfide bonds moves faster in this system than the chymotrypsin trypsin propeptide chimera containing only four interchain disulfide bridges (Fig. 2). It is not evident, however, that why wild-type chymotrypsinogen moves faster in the SDS polyacrylamide gel, than the chymotrypsin trypsin propeptide chimera construction (lanes 4–5 in Fig. 2). This may have something to do with differential proteolytic cleavage(s) of the two proteins (see below).

Our interpretation of the gel electrophoretic patterns as to the different expression levels are concerned was supported by direct enzyme activity measurements of the same yeast culture media (Table 1). Yeast culture media with the highest concentration of expressed proteins, wild-type chymotrypsinogen, the chymotrypsin trypsin propeptide chimera and wild-type trypsinogen (lanes 4–6 in Fig. 2) were found to exhibit the largest protease activities after specific zymogen activation (Table 1). The comparison of the chymotrypsin activities in culture media before and after activation showed that almost 100% of the Cys1Ser–Cys122Ala and Cys1Ser–Cys122Ser chymotrypsinogen mutants were present in active form, in contrast to the 1–2% active protease in the wild-type chymotrypsinogen expressing culture. This ratio was even lower in cultures expressing the chymotrypsin trypsin chimera zymogen and wild-type trypsinogen (Table 1).

To chemically characterize the non-specifically activated forms of chymotrypsinogen mutants Cys1Ser–Cys122Ala and Cys1Ser–Cys122Ser as well as Cys122Ser chymotrypsin released by enterokinase from the chymotrypsin trypsin propeptide chimera, they were purified to homogeneity (see section 2) and subjected to amino acid sequence analysis up to the first five N-terminal amino acid residues. The analyses revealed the presence of two major N-termini in all three preparations, Ile16 that is usually formed by the specific activation procedure and Asn147 (and, in a smaller amount, Leu149) that may result from the autocatalysis of chymotrypsin/ogen [17,18]. In a smaller ratio two further N-termini corresponding to residues 115 (Ser) and 131 (Pro) were also detected. These latter ones may be due to further self cleavages of chymotrypsin. Thus, N-terminal sequence analysis of the purified chymotrypsin mutants provided evidence that their structures do not, at least

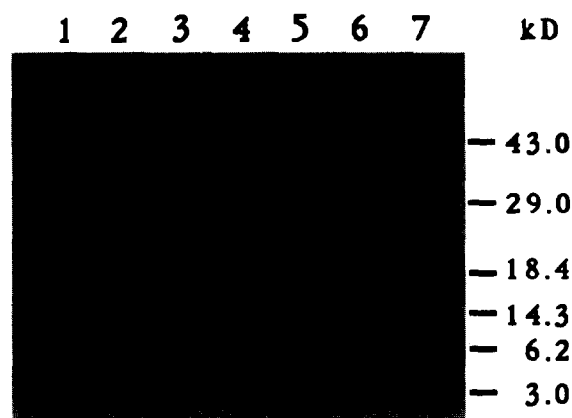


Fig. 2. The expression of the cloned wild-type and mutant enzymes in yeast culture medium analyzed by SDS polyacrylamide gel electrophoresis in the absence of reducing agents. Purified  $\Delta 1-15$ ,Cys122Ser chymotrypsin (1), Cys1Ser,Cys122Ala chymotrypsinogen (2), Cys1Ser,Cys122Ser chymotrypsinogen (3), wild-type chymotrypsinogen (4), chymotrypsin trypsin propeptide chimera chymotrypsinogen (5), wild-type trypsinogen (6).

significantly, depend on the activation procedure, and that all these preparations are devoid of the propeptide cleaved off.

Wild-type chymotrypsin, wild-type trypsin and the two chymotrypsin mutants were purified to homogeneity and their enzyme activities were determined on a series of amide substrates. Their kinetic parameters are presented in Table 2. The data show that the chymotrypsin mutants formed by either non-specific (NS) or enterokinase (E) activation exhibit activities undistinguishable from each other and from those of wild-type chymotrypsin produced from the zymogen by trypsin activation (T) (Table 2). The enterokinase (E) and the non-specifically (NS) activated forms, being not contaminated with any trypsin, allowed a precise measurement of the intrinsic tryptic activity of chymotrypsin (Table 2A).

#### 4. Discussion

Proteolytic enzymes are expressed as zymogens with an N-terminal propeptide sequence of different length. The propeptides maintain the enzymes in their inactive form until they reach the physiological compartment where their activity is required. Additionally, some longer propeptides are necessary for the correct protein folding (e.g. subtilisin E and  $\alpha$ -lytic protease [19]). The relatively short propeptides in trypsinogen

and chymotrypsinogen apparently have only the former function. The activation of these zymogens is triggered by the proteolytic cleavage between the 15th and 16th amino acids [2,3] (see Fig. 1). Activation of trypsinogen by specific cleavage of enterokinase occurs via a five amino acid long recognition sequence, not recognized by other proteases. Thus the trypsinogen propeptide, though it has a completely disordered conformation [4–6], effectively prevents premature activation. In contrast, in chymotrypsinogen, the Arg15–Leu16 sequence is a very typical cleavage site that can be recognized by many proteases other than trypsin (such as Kex2, a membrane-bound protease in yeast that preferentially cleaves arginyl bonds [20]). However, the reduction of the propeptide mobility can also be an alternative to avoid non-specific activation. Indeed, with the exception of residues 11–13, the chymotrypsinogen propeptide has a well-defined conformation [7–9,21] stabilized by five H-bonding interactions and more importantly by the Cys1–Cys122 disulfide bond.

Our results support the view that the sterically restricted structure of the activation site in chymotrypsinogen rather than the propeptide sequence itself is the major factor that prevents premature activation of the zymogen. We have shown that the elimination of the Cys1–Cys122 disulfide bond completely unprotects chymotrypsinogen from non-specific activation in the yeast expression system. As to the nature of the protease(s), responsible for non-specific activation, we suppose that involvement of trypsin like enzyme(s) which is (are) non-secretory (e.g. bound to the yeast cell membrane – see above), because no significant trypsin-like (and chymotrypsin-like) activity was detected in the culture medium of non-transformed yeast (Table 1). However, the quite unlikely possibility can not be completely excluded either, that a very low intrinsic trypsin-like activity of the chymotrypsinogen mutants without disulfide bridge 1–122 causes the non-specific self cleavage of these constructs at the Arg15–Ile16 peptide bond.

It has been shown that the active/zymogen ratio is more than ten-fold lower in the cultures expressing trypsinogen than the ones expressing chymotrypsinogen (Table 1), suggesting that the trypsinogen propeptide is a more potent inactivator of the zymogen. This property of the trypsinogen propeptide has been successfully transferred to chymotrypsinogen by replacing the chymotrypsinogen propeptide with the trypsinogen one. Significantly, the expression level of this chymotrypsin trypsin propeptide chimera is about 10 times higher than that of wild-type chymotrypsinogen (Table 1). Since the catalytic properties and the substrate specificity of  $\Delta 1-15$ ,Cys122Ser chymotrypsin,

Table 1

Chymotrypsin or trypsin activities of the yeast culture media containing different chymotrypsinogen mutants, wild-type chymotrypsinogen and trypsinogen expressed

| Expressed protein                       | Activity in 100 $\mu$ l medium ( $\Delta$ OD <sub>410</sub> $\cdot$ min <sup>-1</sup> ) |                  | Ratio (%) |
|---|---|------------------|-----------|
|   | Before activation   | After activation |           |
| C1S,C122A chymotrypsinogen              | 0.0527  | 0.0571           | 91.20     |
| C1S,C122S chymotrypsinogen              | 0.0400  | 0.0452           | 89.60     |
| Wild-type chymotrypsinogen              | 0.0034  | 0.2512           | 1.35      |
| Chymotrypsin-trypsin propeptide chimera | 0.0022  | 1.6050           | 0.14      |
| Wild-type trypsin                       | < 0.0001  | 0.0810           | < 0.12    |
| Culture medium <sup>+</sup>             | 0.0002  | 0.0003           | –         |
| Culture medium <sup>5</sup>             | 0.0000  | 0.0002           | –         |

<sup>+</sup> Chymotrypsin activity.

<sup>5</sup> Trypsin activity.

Table 2

Kinetic constants determined by the hydrolysis rates of fluorogenic (A) and photometric (B) substrates

Table 2A

| Enzyme                            | Xaa =          | Activities on Succinyl-AlaAlaProXaa-AMC |                   |                      |                      |
|-----------------------------------|----------------|---|-------------------|----------------------|----------------------|
|                                   |                | Leu                                     | Tyr               | Phe                  | Lys                  |
| Wild-type chymotrypsin            | (T) $k_{cat}$  | $8.8 \times 10^2$                       | $6.3 \times 10^3$ | $5.9 \times 10^3$    | –                    |
|                                   | $K_m$          | $3.2 \times 10^1$                       | $1.2 \times 10^1$ | $2.2 \times 10^1$    | –                    |
|                                   | $k_{cat}/K_m$  | $2.7 \times 10^1$                       | $5.3 \times 10^2$ | $2.7 \times 10^2$    | –                    |
| $\Delta 1-15, C122A$ chymotrypsin | (NS) $k_{cat}$ | $5.3 \times 10^2$                       | $6.7 \times 10^3$ | $7.2 \times 10^3$    | $1.6 \times 10^0$    |
|                                   | $K_m$          | $2.5 \times 10^1$                       | $1.6 \times 10^1$ | $2.0 \times 10^1$    | $2.2 \times 10^2$    |
|                                   | $k_{cat}/K_m$  | $2.1 \times 10^1$                       | $4.3 \times 10^2$ | $3.6 \times 10^2$    | $7.5 \times 10^{-3}$ |
| $\Delta 1-15, C122S$ chymotrypsin | (NS) $k_{cat}$ | $4.8 \times 10^2$                       | $6.7 \times 10^3$ | $6.2 \times 10^3$    | $3.7 \times 10^0$    |
|                                   | $K_m$          | $3.3 \times 10^1$                       | $1.6 \times 10^1$ | $2.3 \times 10^1$    | $1.9 \times 10^2$    |
|                                   | $k_{cat}/K_m$  | $1.5 \times 10^1$                       | $4.3 \times 10^2$ | $2.6 \times 10^2$    | $1.9 \times 10^{-2}$ |
| $\Delta 1-15, C122S$ chymotrypsin | (E) $k_{cat}$  | $5.2 \times 10^2$                       | $7.1 \times 10^3$ | $5.3 \times 10^3$    | $4.8 \times 10^0$    |
|                                   | $K_m$          | $4.7 \times 10^1$                       | $1.1 \times 10^1$ | $2.2 \times 10^1$    | $2.2 \times 10^2$    |
|                                   | $k_{cat}/K_m$  | $1.1 \times 10^1$                       | $6.5 \times 10^2$ | $2.4 \times 10^2$    | $2.1 \times 10^{-2}$ |
| Wild-type trypsin                 | (E) $k_{cat}$  | –                                       | –                 | $2.2 \times 10^0$    | $2.3 \times 10^3$    |
|                                   | $K_m$          | –                                       | –                 | $1.6 \times 10^2$    | $5.7 \times 10^{-1}$ |
|                                   | $k_{cat}/K_m$  | –                                       | –                 | $1.4 \times 10^{-2}$ | $3.8 \times 10^3$    |

 $k_{cat}$ , s<sup>-1</sup>;  $K_m$ ,  $\mu$ M;  $k_{cat}/K_m$  s<sup>-1</sup>· $\mu$ M<sup>-1</sup>.

The zymogen activation was made by TPCK-treated trypsin (T), highly purified enterokinase (K) or was non-specific (NS – using no activator enzyme – see the text). The values of 3–5 measurements were averaged.

Table 2B

| Enzyme                            |                | Activities on        |                   |
|-----------------------------------|----------------|----------------------|-------------------|
|                                   |                | Succ.AAPF-pNA        | L-Tyr-pNA         |
| Wild-type chymotrypsin            | (T) $k_{cat}$  | $5.9 \times 10^3$    | $6.1 \times 10^3$ |
|                                   | $K_m$          | $3.1 \times 10^1$    | $4.9 \times 10^1$ |
|                                   | $k_{cat}/K_m$  | $1.9 \times 10^2$    | $1.3 \times 10^2$ |
| $\Delta 1-15, C122A$ chymotrypsin | (NS) $k_{cat}$ | $5.0 \times 10^3$    | $3.7 \times 10^3$ |
|                                   | $K_m$          | $3.0 \times 10^1$    | $3.6 \times 10^1$ |
|                                   | $k_{cat}/K_m$  | $1.7 \times 10^{-2}$ | $1.0 \times 10^2$ |
| $\Delta 1-15, C122S$ chymotrypsin | (NS) $k_{cat}$ | $5.0 \times 10^3$    | $2.8 \times 10^3$ |
|                                   | $K_m$          | $4.3 \times 10^1$    | $2.7 \times 10^1$ |
|                                   | $k_{cat}/K_m$  | $1.9 \times 10^2$    | $1.0 \times 10^2$ |
| $\Delta 1-15, C122S$ chymotrypsin | (E) $k_{cat}$  | $4.7 \times 10^3$    | $4.7 \times 10^3$ |
|                                   | $K_m$          | $6.5 \times 10^1$    | $3.8 \times 10^1$ |
|                                   | $k_{cat}/K_m$  | $7.2 \times 10^1$    | $1.2 \times 10^2$ |

 $k_{cat}$ , s<sup>-1</sup>;  $K_m$ ,  $\mu$ M;  $k_{cat}/K_m$  s<sup>-1</sup>· $\mu$ M<sup>-1</sup>.

The zymogen activation was made by TPCK-treated trypsin (T), highly purified enterokinase (K) or was non-specific (NS – using no activator enzyme – see the text). The values of 3–5 measurements were averaged.

that is the active form of the chymotrypsin trypsin chimera, are identical to these of the wild-type chymotrypsin (Table 2), further structure-activity studies on chymotrypsin are carried out by mutating and expressing the chymotrypsin trypsin propeptide chimera construct described in this paper.

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## References

- [1] Hedstrom, L., Szilágyi, L. and Rutter, W.J. (1992) Science 255, 1249–1253.
- [2] Neurath, H. and Davie, E. (1955) J. Biol. Chem. 212, 515.
- [3] Desnuelle, P. and Fabre, C. (1955) Biochim. Biophys. Acta 18, 49.
- [4] Bode, W., Felhammer, H. and Huber, R. (1976) J. Mol. Biol. 106, 325–335.
- [5] Kossiakoff, A.A., Chambers, J.L., Kay, L.M. and Stroud, R.M. (1977) Biochemistry 16, 654–664.
- [6] Stroud, R.M., Kossiakoff, A.A. and Chambers, J.L. (1977) Ann. Rev. Biophys. Biochem. 6, 177–193.
- [7] Freer, S.T., Kraut, J., Robertus, J.D., Wright, H.T. and Xuong, Ng. H. (1970) Biochemistry 9, 1997–2009.
- [8] Kraut, J. (1970) in: The Enzymes (Boyer, P.D., Ed.) Vol. 3 pp. 165, Academic Press, New York, London.
- [9] Wang, D., Bode, W. and Huber, R. (1985) J. Mol. Biol. 185, 595–624.
- [10] Gráf, L., Jancsó, A., Szilágyi, L., Hegyi, G., Pinter, K., Náray-Szabó, G., Hepp, J., Medzihradsky, K. and Rutter, W.J. (1988) Proc. Natl. Acad. Sci. USA 85, 4961–4965.
- [11] Craik, C.S., Choo, Q.-L., Swift, G.H., Quinto, C., MacDonald, R.J. and Rutter, W.J. (1984) J. Biol. Chem. 259, 14255–14264.
- [12] Kunkel, T.A. (1985) Proc. Natl. Acad. Sci. USA 82, 488–492.
- [13] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
- [14] Phillips, M.A., Fletterick, R. and Rutter, W.J. (1990) J. Biol. Chem. 265, 20692–20698.
- [15] Schellenberger, V., Turck, C.V., Hedstrom, L. and Rutter, W.J. (1993) Biochemistry 32, 4349–4353.
- [16] Jameson, G.W., Adams, D.V., Kyle, W.S. and Elmore, D.T. (1973) Biochem. J. 131, 107–117.
- [17] Kunitz, M. and Northrop, J.H. (1935) J. Gen. Physiol. 18, 433.
- [18] Kunitz, M. (1938) J. Gen. Physiol. 22, 207.
- [19] Abita, J.P., Delaage, M., Lazdunski, M. and Savrda, J. (1969) Eur. J. Biochem. 8, 314–324.
- [20] Brenner, C. and Fuller, R. (1992) Proc. Natl. Acad. Sci. USA 89, 922–926.
- [21] Maroux, S., Baratti, J. and Desnuelle, P. (1971) J. Biol. Chem. 246, 5031–5039.