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# Inhibition of aspartic proteinases by propart peptides of human procathepsin D and chicken pepsinogen

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Two propart peptides of aspartic proteinases, the propart peptide of chicken pepsin and human cathepsin D, respectively, were investigated from the point of view of their inhibitory activity for a set of aspartic proteinases. These peptides display a very broad inhibitory spectrum. The strongest inhibition was observed for pepsin A-like proteinases where propart peptides can be used as titrants of active enzymes.

Aspartic proteinase; Propart peptide; Human procathepsin D; Chicken pepsinogen; Inhibition; Zymogen activation

## 1. INTRODUCTION

Even though aspartic proteinases play an important role in many physiological processes, not many of their natural polypeptide inhibitors have been described yet. The inhibitor isolated from the roundworm *Ascaris lumbricoides* is a potent inhibitor of pepsin-like proteinases as is pepsin A, gastricsin and cathepsin E [1,2]. Another example is the inhibitor $IA_3$ from yeast which is highly specific for vacuolar yeast proteinase A [3]. Recently, primary structures of potato iso-inhibitors of lysosomal aspartic proteinase cathepsin D have been published [4,5]. This type of inhibitor again displays significant singularity of inhibition of aspartic proteinases, inhibiting only cathepsin D.

Fragments of propart peptides of pig pepsinogen were studied [6] and the intact propart activation peptide of chicken pepsinogen has been shown to inhibit chicken pepsin and pig pepsin, two representatives of pepsin A-like proteinases [7].

Inhibitor properties of the two intact propart peptides, chicken pepsinogen propart peptide (CPP) (42 amino acid residues) and human cathepsin D propart peptide (HCDP) [8] (46 amino acid residues), have now been further characterized from the point of view of the inhibitory spectrum for different types of aspartic proteinases.

*Abbreviations:* CPP, chicken propart peptide; HCDP, human cathepsin D propart peptide; Tris, tris-(hydroxymethyl)aminomethane; $F(NO_2)$, *p*-nitrophenylalanyl; PDI, potato tubers cathepsin D inhibitor

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## 2. EXPERIMENTAL

### 2.1. *Materials*

*Enzymes.* Chicken pepsin was obtained according to [9]. Bovine spleen cathepsin D was isolated according to [10], calf chymosin was obtained from Prof. B. Foltman (The Institute of Biochemical Genetics, University of Copenhagen, Denmark), human gastricsin and human pepsin A were obtained from Dr. Z. Kučerová (Charles University, Prague, Czechoslovakia), pig pepsin was a product of Worthington (New Jersey, USA), *Endothia parasitica* aspartic proteinase and *Mucor pusillus* proteinase were obtained from Dr. S. Foundling (Du Pont, Wilmington USA), penicillopepsin proteinase was obtained from Dr. T. Hoffman (University of Toronto, Canada).

*Propart peptides.* Chicken propart peptide was isolated as it was described previously [7], human cathepsin D propart peptide synthesis was accomplished by the solid-phase multiple peptide synthesis under low-pressure continuous-flow conditions using a manually operated synthesizer. The synthesis was carried out in a flow reactor with adjustable volume using $F_{moc} \times t$Bu protection strategy on standard methylbenzhydrylamine polystyrene-based resin [11]. The purity of both peptides was confirmed by amino acid analysis (Durrum 500 amino acid analyser) and by sequencing (Applied Biosystems, model 470A sequencer).

*Substrates:* K P A E F $F(NO_2)$ A L:S1, Ac A A A K $F(NO_2)$ A A:S2, and H P H P L S $F(NO_2)$ M A I P P K K:S3 were kindly donated by Dr. P. Štrop (Institute of Organic Chemistry and Biochemistry, Prague, Czechoslovakia).

### 2.2. *Determination of* $K_i$ *values*

For the determination of inhibition constants, a spectrophotometric assay was used. The initial velocities were determined by monitoring the change of the absorbance caused by cleavage of synthetic substrates at 305 nm [12]. Buffers used for assays were 0.1 mol·l$^{-1}$ acetate buffers, buffers used for preincubation at pH higher than 7 were 0.05 mol·l$^{-1}$ Tris-HCl. From recorded initial velocity data the $K_i$ values were determined by Dixon plot [13] or by measuring the $IC_{50}$ values and using the equation:

$$K_i = (IC_{50} - E_t/2)/(1 + S/K_m)$$

where $E_t$ is the total concentration of active enzyme in assay and $S$ is the initial substrate concentration [14].



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Table I

$K_i$ values for the interaction of chicken pepsinogen propart peptide with different aspartic proteinases

| Enzyme | pH 1 | pH 2 | $K_i$ [μM] | Substrate |
|---|---|---|---|---|
| Chicken pepsin | 7.5 | 5.9 | <0.01 | S1 |
| Pig pepsin | no | 5.5 | 0.02 | S1 |
| Pig pepsin | 5.5 | 5.5 | 0.001 | S1 |
| Cathepsin D | 7.4 | 5.5 | >1000 | S1 |
| Human pepsin A | 5.5 | 5.5 | 0.001 | S1 |
| Human gastricsin | 5.75 | 5.75 | 0.04 | S1 |
| Calf chymosin | 7.2 | 6.2 | 0.10 | S3 |
| Penicillopepsin | 6.0 | 6.0 | 0.2 | S2 |
| *Endothia* pepsin | no | 6.0 | 0.03 | S2 |
| *Endothia* pepsin | 6.0 | 6.0 | >600 | S2 |
| *Mucor pusillus* pepsin | no | 6.0 | 0.8 | S2 |

Symbols: pH 1, pH of the preincubation, pH 2, pH of the kinetic assay.

Table II

$K_i$ values for the interaction of human cathepsin D propart peptide with different aspartic proteinases

| Enzyme | pH 1 | pH 2 | $K_i$ [μM] | Substrate |
|---|---|---|---|---|
| Cathepsin D | 7.4 | 5.5 | 0.03 | S1 |
| Cathepsin D | 5.5 | 5.5 | 10.0 | S1 |
| Chicken pepsin | 7.5 | 5.9 | 0.13 | S1 |
| Chicken pepsin | no | 5.9 | 2.35 | S1 |
| Pig pepsin | 5.5 | 5.5 | 0.005 | S1 |
| Human pepsin A | | | n.d. | |
| Human gastricsin | | | n.d. | |
| Calf chymosin | | | n.d. | |
| Penicillopepsin | 6.0 | 6.0 | 15.0 | S2 |
| *Endothia* pepsin | no | 6.0 | 1.0 | S2 |
| *Endothia* pepsin | 6.0 | 6.0 | 54.0 | S2 |

Symbols: pH 1, pH of the preincubation; pH 2, pH of the kinetic assay; n.d., not determined.

## 3. RESULTS AND DISCUSSION

As can be seen in Tables I and II, the inhibitory spectrum of both propart peptides is surprisingly broad. Both interact significantly with all enzymes tested. In contrary to other inhibitors (PDI, *Ascaris*) the binding of propart peptides is shifted toward a higher pH. This is in accordance with their natural function in activation processes but the pH shift highly complicates the determination of the inhibitory constants. To make it possible to determine the association constants we often used preincubation of the mixture of the enzyme with the propart peptide at higher pH to allow to form an inhibitory complex.

As follows from Table I, CPP has the highest affinity to gastric pepsins of type A (pig pepsin, human pepsin A) and to the parent enzyme. Interestingly no inhibition by CPP was found for cathepsin D. For HCDP (Table II) the inhibition spectrum is generally similar to that of CPP, again for pig pepsin the $K_i$ value is in the nanomol range. Contrary to CPP behaviour to cathepsin D, it was found that HCDP inhibits chicken pepsin. From their kinetic behaviour and the comparison of $K_i$ values received with or without preincubation at a pH higher than neutral, it is clear that slow tight binding of the propart molecules to enzymes takes place, probably leading to a conformation similar to that of zymogens.

When the pH is lowered by adding the complex formed at higher pH to the assay mixture, complicated behaviour is observed. In these cases (e.g. complexes of chicken pepsin or of cathepsin D with their own propart peptides) the initial velocity expresses the tight binding interaction, while after several minutes this complex changes to a different one characterized by lower in-

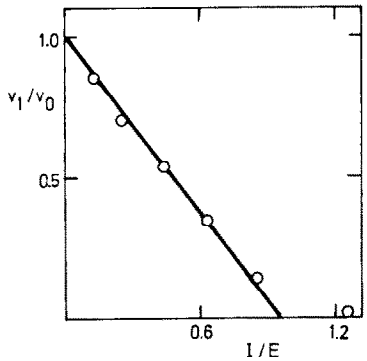


Fig. 1. Titration of chicken pepsin by CPP at pH 7.5. The enzyme ($1\times10^{-7}$ mol·l$^{-1}$) was preincubated for 30 min with CPP (0–$1.23\times10^{-7}$ mol·l$^{-1}$) at pH 7.5 at 25°C. The fractional residual activity was determined at pH 5.9 using substrate S1, making use of the fact that the complex breakdown at pH 5.9 is slower than the time necessary for the determination of the residual activity. $v_i/v_o$ is the ratio of initial velocity of the enzyme preincubated with CPP and initial velocity of the enzyme preincubated without any inhibitor; $I/E$ is the ratio of the molar content of CPP to that of the enzyme.

hibition. Initial velocities were used for the determination of $K_i$ values.

The interaction of CPP and HCDP with fungal aspartic proteinases was highly different. In this case, according to kinetic behaviour and HPLC studies (data not shown), both propart peptides behave as very tightly bound substrates (the $K_m$ lower than $10^{-7}$) [15] and only after their consumption the true inhibition is observed, which is accomplished by fragments of propart peptides. This is also in accordance with different specificities of fungal aspartic proteinases [16].

Overall, we can conclude that the propart peptides described in this paper are strong inhibitors of aspartic proteinases and they can be used for the titration of aspartic proteinases of type pepsin A and cathepsin D (Fig. 1). Moreover, understanding the mechanism of the interaction of aspartic proteinases with propart peptides allows for a deeper insight into the process of activation of their zymogens, since the artificial complex formed at higher pH can mimic an aspartic proteinase zymogen [7].

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