PROTEASES

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The spontaneous cleavage of a peptide bond occurs at a very slow rate

Comparison of uncatalyzed (uncat) and catalyzed (cat) reactions:
• carboxypeptidase B (hydrolysis of a terminal peptide bond)
• angiotensin-converting enzyme (hydrolysis of an internal bond)
• ascites tumor dipeptidase (hydrolysis of a dipeptide)

<table>
<thead>
<tr>
<th></th>
<th>Half-time (years)</th>
<th>$k_{\text{uncat}}$ (s$^{-1}$)</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$k_{\text{cat}}/k_{\text{uncat}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exopeptide</td>
<td>1100</td>
<td>$1.8 \times 10^{-11}$</td>
<td>238.0</td>
<td>$1.3 \times 10^{13}$</td>
</tr>
<tr>
<td>Endopeptide</td>
<td>19</td>
<td>$1.1 \times 10^{-9}$</td>
<td>13.9</td>
<td>$1.2 \times 10^{10}$</td>
</tr>
<tr>
<td>Dipeptide</td>
<td>15</td>
<td>$1.5 \times 10^{-9}$</td>
<td>1842.0</td>
<td>$1.2 \times 10^{12}$</td>
</tr>
</tbody>
</table>


Enzymes enhance the rate by 10-13 orders of magnitude!
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Chymotrypsinogen, 245 Amino Acids

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGVPAIQPVL SGLSRIVNGE EAVPGSWPWQ VSLQDKTGFH FCGGSLINEN WVVTAAHCGV</td>
<td>60</td>
</tr>
<tr>
<td>TTSDDVVGAGE FDQGSSSEKI QKLKIAKVFK NSKNLSLTIN NDITLLLKLST AASFSQTVSA</td>
<td>120</td>
</tr>
<tr>
<td>VCLPSASDDDF AAGTTCVTTG WGLTRYTNAN TPDLQQASL PLLSNTNCKK YWGTKIKDAM</td>
<td>180</td>
</tr>
<tr>
<td>ICAGASGVSS CMGDSGGPLV CKKNGAWTLV GIVSWGSSTC STSTPGVYAR VTALVNWVQQ</td>
<td>240</td>
</tr>
<tr>
<td>TLAAN</td>
<td></td>
</tr>
</tbody>
</table>

Cleavage of:
S14-R15
T147-N148

Mature enzyme

Chymotrypsin, 241 Amino Acids

<table>
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<th>Position</th>
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</thead>
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<tr>
<td>CGVPAIQPVL SGLIVNGE EAVPGSWPWQ VSLQDKTGFH FCGGSLINEN WVVTAAHCGV</td>
<td>58</td>
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<tr>
<td>TTSDDVVGAGE FDQGSSSEKI QKLKIAKVFK NSKNLSLTIN NDITLLLKLST AASFSQTVSA</td>
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<tr>
<td>VCLPSASDDDF AAGTTCVTTG WGLTRY AN TPDLQQASL PLLSNTNCKK YWGTKIKDAM</td>
<td>176</td>
</tr>
<tr>
<td>ICAGASGVSS CMGDSGGPLV CKKNGAWTLV GIVSWGSSTC STSTPGVYAR VTALVNWVQQ</td>
<td>236</td>
</tr>
<tr>
<td>TLAAN</td>
<td></td>
</tr>
</tbody>
</table>

Chymotrypsin:
- 3 N-termini
- 3 C-termini
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Mature Chymotrypsin

Chain C (blue)

Chain B (purple)

Chain A (cyano)
Disulfide bridges hold the chains together

α-chymotrypsin:

• 10 cysteines
• Chain A (1-13), 1 cysteine
• Chain B (16-146), 4 cysteines
• Chain C (149-245), 5 cysteines

• Chain A linked to chain B by 1 disulfide bridge
• Chain B linked to chain C by 1 disulfide bridge
• 3 intra-chain disulfide bridges
• 1 bridge in chain B
• 2 bridges in chain C
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Inter-chain and intra-chain disulfide bridges
The catalytic triad of α-chymotrypsin

3 essential residues:

- H57 (acid-base catalysis)
- D102 (charge stabilization)
- S195 (nucleophile)

- Histidine increases the acidity of serine –OH (nucleophile)
- Serine attacks the carbonyl
- Aspartate stabilizes the charge (positive) on Histidine

Concerted action of 3 residues
Distances and orientation among residues: peculiarity of S195
Isoleucine16 moves towards the active site
Histidine 57: base catalysis

The –OH of serine is a poor nucleophile. Histidine 57, acting as a base, generates a strong nucleophile (–O\(^{-}\)) and gets a positive charge. Aspartate 102 stabilizes the charge on Histidine 57.
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Oxyanion hole

Upon the nucleophilic attack by S195:

- the carbonyl C is transiently tetrahedral
- the C-O group bears a negative charge (oxyanion)

The active site of α-chymotrypsin stabilizes the oxyanion. This stabilization is fulfilled by the “oxyanion hole” (G193-S195). The oxyanion hole is located between 2 β strands.
The negative charge on C-O is stabilized by interactions with G193 and S195 NH Groups (backbone).

Oxyanion hole
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3

4

5

6
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Free enzyme is regenerated
Hydrophobic residues of α-chymotrypsin

Hydrophobic residues: red.
Hydrophilic residues: blue.

A hydrophobic pocket is located near S195. This pocket confers specificity to α-chymotrypsin. Peptide bonds hydrophobic-X or aromatic-X are preferentially cleaved (the hydrophobic amino acid is located at the N-terminal side of the peptide unit). Trypsin (also a seryl-protease) cleaves R-X peptide units.
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The aromatic ring of substrate’s F, Y, or W interacts with the hydrophobic pocket of α-chymotrypsin. Large hydrophobic groups can also be accommodated. The C’ of aromatic amino acids is subjected to nucleophilic attack by α-chymotrypsin.

α-chymotrypsin is also able to hydrolyze ester bonds between an aromatic moiety (e.g. p-nitrophenol) and an acid (e.g. acetic acid). p-nitrophenylacetate is readily hydrolyzed by α-chymotrypsin and was used as a model substrate to investigate in detail the kinetics of α-chymotrypsin-catalyzed reactions.
The regeneration of free enzyme starting from acyl-chymotrypsin is rate-limiting. The enzyme binds a water molecule, and H57 acts as a base: the nucleophilicity of H_2O is increased and C=O is attacked. A poor leaving group (R_1-COOH) has to be released in order to regenerate free α-chymotrypsin.

How can be demonstrated the slow rate of enzyme deacylation?

• difficult task with proteins or peptides as substrates; the first (P1) and the second (P2) reaction product should be distinguished. P1 and P2 are the N-ter and the C-ter fragments of the substrate, respectively.
• usefulness of a substrate the cleavage of which yields a spectrophotometrically-detectable product (P1 or P2).
p-nitrophenolate (pNP) absorbs visible light. The release of pNP by α-chymotrypsin can be conveniently monitored at 420 nm. When the release of pNP occurs, enzyme is acylated. Free enzyme must be regenerated to perform a second catalytic cycle. Further pNP will be released depending on the [E] in the free form.
The “burst” in the presence of p-nitrophenyl-ethylcarbonate

**Assay mixture:**

- 66 mM Pi buffer, pH 7.6
- 0.5 mM PNC
- \([E] = 0.1-0.8 \text{ mg/mL}\)
- \(M_r \alpha\text{-chymotrypsin} = 25.2 \text{ kDa}\)
- \([E] = 4-32 \mu\text{M}\)

During the first 2-3 minutes, a rapid (“burst”) release of p-nitrophenolate occurs. The concentration of p-NP released is proportional to enzyme concentration. Afterwards, a slower kinetics is observed.

The “burst” in the presence of p-nitrophenyl-ethylcarbonate

In the presence of p-nitrophenyl acetate as substrate the initial “burst” is also observed.

The regeneration of free enzyme from the acylated form is rate-limiting. Mechanism?

During the first 2-3 minutes a consistent amount of pNP is produced. Later on, the reaction proceeds at a slower, constant velocity. The amplitude of the fast phase is strictly proportional to the concentration of α-chymotrypsin.

Enzyme deacylation is rate-limiting.
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α-chymotrypsin and pH

The velocity of the reaction catalyzed by α-chymotrypsin is maximal at pH 8.

Reaction velocity as a function of pH is bell-shaped

- $k_{\text{cat}}$ increases as a function of pH
- $K_m$ increases as a function of pH
- $k_{\text{cat}}$ vs. pH suggests that the base His57 is necessary to prime the catalytic cycle
- $K_m$ vs. pH suggests that a basic group (amino) is also essential for the binding of substrate

Nature of the group affecting $K_m$?
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ChainB-ChainC interaction

Catalytic triad
A salt bridge is present between Ile16 (N-ter) and Asp194

The N-ter of Ile16 features a pKa equal to 9.1. Below this pH the positively charged form of Ile16 amino group is favoured. The ionic interaction with Asp194 is possible.
Complex between α-chymotrypsin and inhibitor from *Locusta migratoria*

*L. migratoria* inhibitor (purple)

α-chymotrypsin (cyano)
Positioning of the β-strand bearing W215
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