e-PG Pathshala

Paper 05 **:** Molecular Enzymology and Protein Engineering
Module 04 **:** Mechanism of action of Trypsin, Ribonuclease and carboxypeptidase
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Objective:

Enzyme in the cell pays important role in the physiology of the cells. The objective of the current module is to understand the mechanism of action of Trypsin, Ribonuclease and carboxypeptidase. This module is divided into following sections-

1. Introduction

Substrate molecule is converted into the product by the enzyme. The rate of conversion of the substrate into the product is slow because of thermodynamic and kinetic barrier that is present to facilitate the reaction. Transition state is the state observed during the chemical reaction having highest free energy and minimal stability. The specific form of the transition state depends on the mechanisms of the particular reaction. Most of the reactions are slow because the substrate does not have sufficient energy to cross the activation energy barrier or transition energy barrier. Catalyst and enzyme facilitates the substrate towards product conversion. Enzymes are biocatalysts that have efficiency, specificity and mode of regulation. In the present module, we will discuss mechanism of action of three enzyme i.e. trypsin, carboxypeptidase and ribonuclease.

2. Trypsin

Trypsin is a pancreatic serine protease enzyme of family S1 that hydrolyses protein with substrate specificity based upon positively charged lysine and arginine side chains. The enzyme is excreted by the pancreas and takes part in the digestion of dietary proteins and other biological processes. It is a medium sized approximately 25kDa globular protein and is produced as an inactive proenzyme trypsinogen. Isoelectric point values vary widely for different forms of trypsin. Both the cationic and anionic form of the enzyme may exist in many organisms. Trypsin and its proenzyme do not undergo any post-translation modifications except proteolytic processing required for its activity.

Trypsinogen is activated by the removal of a terminal hexapeptide to produce single chain β-trypsin in which the catalytic residues bridge two β-barrel domains. Subsequent limited autolysis gives other active forms having two or more peptide chains bound by disulfide bonds. There are two predominant forms namely α-trypsin, having two peptide chains and β-, a single chain. Both show different activity and thermal stability.

Other structural features include surface loops at amino acids 185-193, which influence specificity, despite not making direct contact with the substrate. A high affinity Ca^{2+} binding site is required for stability, and when not present, autolysis occurs. This site is formed by loop Glu70-Glu80. The protein has 6 conserved disulfide bonds at positions 15-145, 33-49, 117-218, 124-191, 156-170, and 181-205. The autolysis loop (located at amino acids 143-151) is very flexible in both trypsin and trypsinogen. Cleavage of this loop at the lysine 145 produces the alpha form which retains some catalytic activity.

The active site residues also known as catalytic triad of trypsin consist of His57, Asp102, Ser195 and it functions at optimal pH of 7.5-8.5. Ser195 acts as a nucleophile in the cleavage reaction, producing an acyl enzyme intermediate. His57 is thought to act as a general base. Asp102 is believed to stabilize the correct tautomer of His57, and to provide compensation for the developing positive charge during the catalysis. Replacement of Asp with Asn result in a 10^4 fold less efficient enzyme than the wild type enzyme in presence of synthetic substrates with an aminomethylcoumarine leaving group, whereas relocating Asp102 to 214 yields a protease that possess 0.5% activity of the wild type enzyme activity on peptide substrates. His57Ala or Ser195 mutants exhibited 105-106 fold less activity than the wild type enzyme on synthetic substrates with an aminomethylcoumarine leaving group. The residual activity in these mutants is due other structural features in the protease which help to stabilize the tetrahedral intermediate.

2.1 Substrate specificity of Trypsin

Trypsin, an endopeptidase cleaves peptides on the C-terminal side of lysine and arginine amino acid residues. If a proline residue is on the carboxyl side of the cleavage site, the cleavage will not occur. If an acidic residue is on either side of the cleavage site, the rate of hydrolysis has been shown to be slower. The process is commonly referred to as trypsin proteolysis or trypsinisation, and proteins that have been digested/treated with trypsin are said to have been trypsinized.

2.2 Isomers of trypsin

Bovine pancreas expresses two forms of trypsin, the dominant cationic and minor anionic forms. These protein sequences share 72% identity, while their coding regions share 78% identity. Each of these proteins is further processed into alternate forms. Catalytic trypsin contains a flexible “autolysis loop” (residues G145-V157), and autolysis of the dominant, single-chain form β-trypsin at K148-S149 within this loop leads to the formation of α-trypsin. Further autolysis at K193-D194 leads to the formation of Psi-trypsin.

Both the cationic and anionic trypsin proteins are expressed as trypsinogen proenzymes, with a 15-residue signal peptide (M1-A15) and an 8-residue propeptide (F16-K23). The three-dimensional fold of all known trypsins is highly conserved. In addition, the catalytic triad and regions flanking the catalytic triad are highly conserved.

2.3 Mechanism of action of trypsin

This serine protease is known to cleave the peptide bonds C-terminal to arginine or lysine. The preference for these basic side chains is evident from the relative values for catalytic efficiency (k_{cat}/k_m) 10^5 greater than natural amino acids. Preference for Arg over Lys is 2 to 10 fold. In small substrates, formation of acyl-enzyme intermediate is the rate determining step for the cleavage of peptide or amide bonds by trypsin and the hydrolysis of this intermediate is the rate limiting step for ester cleavage. In fact, the acylation rate with a substrate is the major specificity determinant and hence, trypsin is much more promiscuous with ester substrates than with peptides. For protein substrates, binding event may be the rate determining step.

The mammalian propeptide (usually a hexapeptide) trypsinogen, possess the consensus sequence for cleavage by the endopeptidase. The cleavage of propeptide leads to disruption of hydrogen bond between His40 and Asp194, which is followed by rotation of Asp194 so that it interacts with the new N-terninus at Ile16. This conformational change results in the formation of oxyanion hole (comprising backbone amides of Gly193 and Ser195) and the

binding pockets. Stabilization of the new conformation in the active domain is due to hydrophobic interactions of the Ile16 side chain. In the other regions of the protein small changes in the positions are made during the process of activation.

The substrate forms a β-antiparallel sheet with the protein site. Substrate specificity is determined by the Asp189 side chain which lies in the bottom of binding pocket. High concentration of acetate increase the catalytic efficiency of the variant enzyme by 300 fold, demonstrating the negative charge at the base of trypsin specificity pocket may be bound with some non-covalently bound ligand.

The cleavage reaction involves two steps. The first step produces a covalent bond between C of the substrate and the hydroxyl group of a reactive Ser residue of the enzyme. The acyl-enzyme intermediate thus produced proceeds through a negatively charged transition state intermediate where bonds of C have tetrahedral geometry instead of planar triangular geometry of the peptide bond. In this step peptide bond is cleaved, one peptide product is attached to the enzyme in the acyl-enzyme intermediate, and the other peptide product rapidly diffuses away. Second step is deacylation where the acyl-enzyme intermediate is hydrolyzed by a water molecule to release the second peptide product with a complete carboxy terminus and to restore the Ser-hydroxyl of the enzyme. This step also proceeds through a negatively charged tetrahedral transition state intermediate.

3. Carboxypeptidase

Carboxypeptidases are zinc containing enzymes that catalyze the hydrolysis of polypeptides at the C-terminal peptide bond. These are secreted as zymogens by the acinar cells of the pancreas. The zymogens are activated by trypsin in the small intestine.

Biochemical and X-ray studies have revealed that zinc atom is essential for catalysts by binding to the carbonyl oxygen of the substrate. This binding weakens the C'=O bond by abstracting electrons from the carbon atom and thus facilitates cleavage of the adjacent peptide bond. It is a large single domain structure comprising a mixed β-sheet of eight β strands with α-helices on both sides. Some of the loop regions are very long and curl around the central theme of the structure.

The four central strands of the β sheet are parallel and have the strand order 8 5 3 4. The strand order is in reverse once, and there is a switch point in the middle of this β sheet between β strands 5 and 3 where the active site is expected to be located.

This is the location where catalytically essential Zn atom is found. Zinc atom is present at this switch point, where it is firmly anchored to the protein by three side chain ligands, His 69, Glu

72, and His 196. The last residue of the β strand 3 is residue 66, so the two zinc ligands His 69 and Glu 72 are at the beginning of the loop region that connects this β strand with its respective α helix. The last residue of the β strand 5 is the third zinc ligand, His 196.

In the structure of carboxypeptidase the loop regions adjacent to the switch point do not provide a binding crevice for the substrate but instead accommodate the active site zinc atom. This zinc atom and the active site are in the predicted position outside the switch point for the four central parallel β strands, even though these β strands are only a small part of the total structure. This sort of arrangement, in which the an active site formed from parallel β strands is flanked by anti parallel β strands, has been found in a number of other α/β proteins with mixed β sheets.

Carboxypeptidase B (CPDB) is a metallocarboxypeptidase that catalyzes the hydrolysis of the basic amino acids, lysine, arginine, and ornithine from the C-terminal position of polypeptides:

Peptidyl-L-lysine (-L-basic amino acid) + H_2O --> Peptide + L-lysine(L-basic amino acid)

CPDB is highly specific for lysine and arginine, but shows greater activity for arginine. It can also act (at a slower rate) on valine, leucine, isoleucine, asparagine, glycine, and glutamine. The differences in specificities between carboxypeptidase A (CPDA) and CPDB can be attributed to the residues Ser 205, Gly 241, and Asp 253 in CPDB as compared to Gly 207, Ile 243, and Ile 255 in CPDA.

The major form of CPDB is found in the monomeric state. CPDB contains 1 atom of zinc per mole of protein. The residues coordinating the zinc residue are conserved and include two histidines, a glutamic acid, and a water molecule. The active site residues include Glu378 and Tyr 356.

4. Ribonuclease

Ribonuclease or RNase is a type of nuclease that catalyzes the degradation of RNA into smaller fragments. It is sub-divided into exoribonuclease and endoribonuclease and comprises of several sub classes within EC 2.7 (for the phosphorolytic enzymes) and 3.1 (for the hydrolytic enzymes) classes of enzymes. Major endoribonuclease includes RNase A, RNase H, RNase III, RNase L, RNase P, RNase Phy M, RNase T1, RNase T2, RNase V, and RNase U2. Exoribonuclease includes RNase PH, polynucleotide phosphorylase (PNPase), RNase R, RNase D, RNase T, oligoribonuclease, exoribonuclease I, and exoribonuclease II.

Endoribonuclease RNase A or pancreatic ribonuclease was the first enzyme and third protein for which a correct amino acid sequence was determined. It is a small protein with molecular weight 13.7 KDa, the mature enzyme only having 124 amino acid residues, with no

carbohydrate attached. RNase A contains 19 of the 20 amino acids, lacking only tryptophan. The three dimensional structure of RNase A is fully encoded by its amino acid sequence. All eight human RNase A-like genes are located on chromosome 14. Each encodes a secretory signal sequence and contains an invariant catalytic triad of two histidines and one lysine with a conserved motif (CKXXNTF). The amino acid sequences of many RNase A homologues have been identified. From the sequences and their distribution over a range of species it has been established that RNase A is a modern protein that is evolving rapidly.

The shape of the protein resembles a kidney, with the active site residues lying in the cleft. The secondary structure has long four-stranded anti-parallel beta-sheets and three short alpha-helices. RNase A is known to have four disulfide bonds, which are essential for the stability of the native enzyme. Two of these disulfide bonds lie between an alpha-helix and a beta-sheet and contribute more to the thermal stability than do the other two. RNase B is a glycoprotein containing at Asn 34 a single oligosaccharide composed of six residues of mannose and two residues of N-acetylglucosamine.

It is known to catalyze the cleavage of the phosphodiester bond between the 5'-ribose of a nucleotide and the phosphate group attached to the 3'-ribose of an adjacent pyrimidine nucleotide. This cleavage forms a 2', 3'-cyclic phosphate, which is then hydrolyzed to the corresponding 3'-nucleoside phosphate. RNase is found in greatest quantity in ruminant pancrease. The major component of the crystalline enzyme is RNase A; a minor component is RNase B. RNase B is the glycosylated form of RNase A.

4.1 Specificity of the Ribonuclease

This enzyme is specific for pyrimidine nucleoside linkages. The reaction is believed to take place in two steps. In the first step, the 3', 5'-phosphodiester bond is cleaved, while generating a 2', 3'-cyclic phosphodiester intermediate. In the second step, the cyclic phosphodiester is hydrolyzed to a 3'-monophosphate group. While the first step is nonspecific with respect to the nitrogenous base of the substrate; but, the second step is very specific for pyrimidine nucleotides with terminal 2', 3'-cyclic phosphates. RNase B has the same specificity as RNase A toward both cyclic cytidylate and yeast RNA. RNase A shows a preference for larger substrates.

The enzyme cleaves at cytidine residues twice as fast as at uridyl residues. Thr45 has been found to be most important for mediating the pyrimidine specificity, both by forming hydrogen bonds with pyrimidine bases and sterically excluding purine bases. The side chain of Asp83 is

important for stabilizing the transition state during the cleavage of uridine-containing substrates; this residue has no effect on the kinetics of cytidine cleavage.

5. Summary

In the present module, we have discussed the mechanism of action of trypsin, chymotrypsin, and ribonuclease enzyme. We have also discussed about the specific amino acid (Trypsin and carboxypeptide) or nucletotide (Ribnuclease) requirement of different enzyme. Trypsin, an endopeptidase cleaves peptides on the C-terminal side of lysine and arginine amino acid residues if proline residue is not present at carboxyl side of the cleavage site. Carboxypeptidase B, catalyzes the hydrolysis of basic amino acids, lysine, arginine, from the C-terminal end of polypeptides. Ribonuclease catalyses the cleavage of the phosphodiester bond between the 5'-ribose of a nucleotide and the phosphate group attached to the 3'-ribose of an adjacent pyrimidine nucleotide. This module will help to study diverse enzyme kinetics of different enzyme.