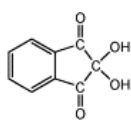


### Amino Acid Composition Determination



ninhydrin

- A peptide can be hydrolyzed into its constituent amino acids by heating in 6N HCl at 110°C for 24 hours
- Amino acids in hydrolysates can be separated by chromatography and quantitated by reacting them with *ninhydrin*
- Ninhydrin treatment yields an intense blue colour, except for proline, which gives a yellow colour because it contains a secondary amino (imino) group

#### 1. Hydrolysis

A known amount of internal standard (norleucine) is added to the sample. Since norleucine does not naturally occur in proteins, is stable to acid hydrolysis and can be chromatographically separated from other protein amino acids. The molar amount of internal standard should be approximately equal to that of most of the amino acids in the sample. The sample, containing at least 5 nmoles of each amino acid (i.e. 10 µg of protein) is then transferred to a hydrolysis tube and dried under vacuum. The tube is placed in a vial containing 6 N HCl and a small amount of phenol and the protein is hydrolyzed by the HCl vapors under vacuum. The hydrolysis is carried out for 65 minutes at 150 deg. C. Following hydrolysis, the sample is dissolved in distilled water containing EDTA (to chelate metal ions) and approximately 1 nmole of each amino acid is placed on a glass amino acid analyzer sample slide.

### Hydrolysis can have varying effects on different amino acids

Valine, isoleucine	Bonds are not easily broken
Threonine, serine	Slowly destroyed by acid hydrolysis.
Methionine	Partially oxidized during acid hydrolysis.
Asparagine, glutamine	Converted to aspartic acid and glutamic acid.
Tryptophan	Completely destroyed by acid hydrolysis.
Cystine	Destroyed by acid hydrolysis.

#### 2. Derivatization

The free amino acids cannot be detected by HPLC unless they have been derivatized. Derivatization is performed automatically on the amino acid analyzer by reacting the free amino acids, under basic conditions, with phenylisothiocyanate (PITC) to produce phenylthiocarbamyl (PTC) amino acid derivatives.

This process takes approximately 30 minutes per sample. A standard solution containing a known amount (500 pmol) of 17 common free amino acids is also loaded on a separate amino acid analyzer sample spot and derivatized.

This will be used to generate a calibration file that can be used to determine amino acid content of the sample. Following derivatization, a methanol solution containing the PTC-amino acids is transferred to a narrow bore HPLC system for separation.

#### 3. HPLC separation

The PTC-amino acids are separated on a reverse phase C18 silica column and the PTC chromophore is detected at 254 nm. All of the amino acids will elute in approximately 25 minutes.

The buffer system used for separation is 50 mM sodium acetate pH 5.45 as buffer A and 70% acetonitrile/32 mM sodium acetate pH 6.1 as buffer B. The program is run using a gradient of buffer A and buffer B with an initial 7% buffer B concentration and ending with a 60% buffer B concentration at the end of the gradient

#### 4. Data interpretation and calculations

Chromatographic peak areas are identified and quantitated using a data analysis system that is attached to the amino acid analyzer system.

A calibration file is used that is prepared from the average values of the retention times (in minutes) and areas (in Au) of the amino acids in 10 standard runs. Since a known amount of each amino acid is loaded onto the analyzer, a response factor ((Au/pmol) can be calculated. This response factor is used to calculate the amount of amino acid (in pmols) in the sample. The amount of each amino acid in the sample is calculated by dividing the peak area of each (corrected for the differing molar absorptivities of the various amino acids) by the internal standard (norleucine) in the chromatogram and multiplying this by the total amount of internal standard added to the original sample.

Mole percent represents the amount of each amino acid present as a percentage of the total amino acids recovered in the sample. Mole percent can be useful for samples in which there is no known composition or molecular weight, non-specific molecular weights, or the sample contains mixtures of proteins, free amino acids and other components.

- $[\text{pmol of individual amino acid}] / [\text{total pmol of all amino acids in the sample}] \times 100 = \text{mole percent of each amino acid}$
- Composition by molecular weight can be used when the molecular weight of the sample is known and the amino acid composition is desired.
- $[\text{pmol of amino acid}] \times [\text{residue molecular weight of amino acid}] = \text{picogram of amino acid}$
- $[\text{picogram of all amino acids}] / [\text{pmol of sample recovered}] = \# \text{ of residues of amino acid per molecule of sample}$
- Composition by residue is used when the amino acid composition is required and the number of times a particular amino acid residue occurs in the sample is known.
- $[\text{pmol of selected amino acid}] / [\text{known \# of residues of selected amino acid/sample molecule}] = \text{pmol of sample recovered}$
- $[\text{pmol of amino acid}] / [\text{pmol of sample recovered}] = \# \text{ of residues of amino acid per molecule of sample}$

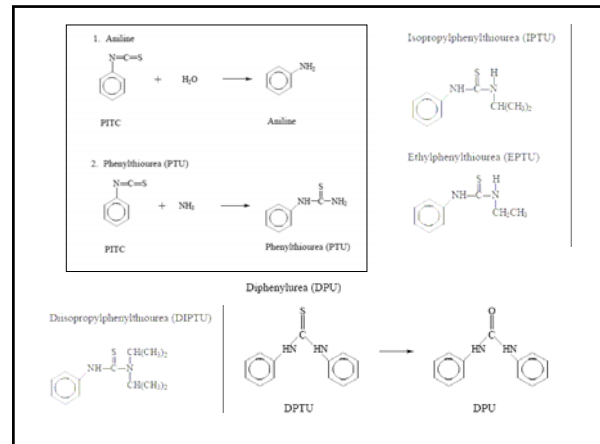
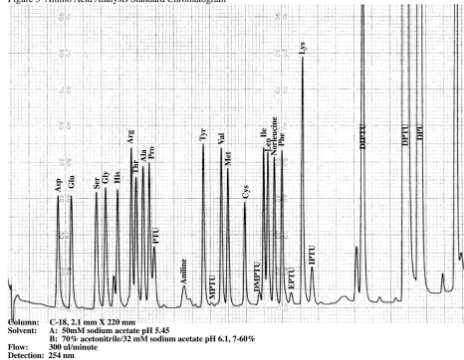
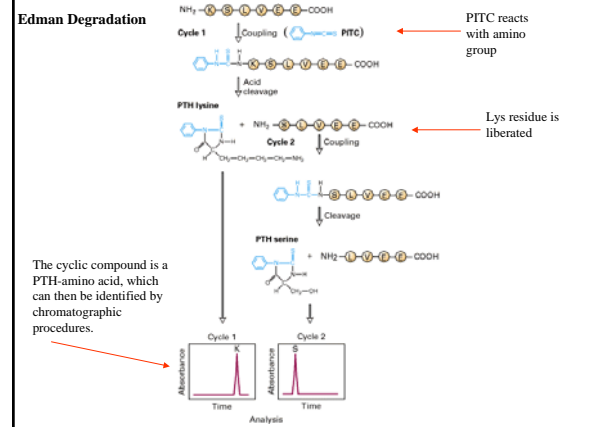
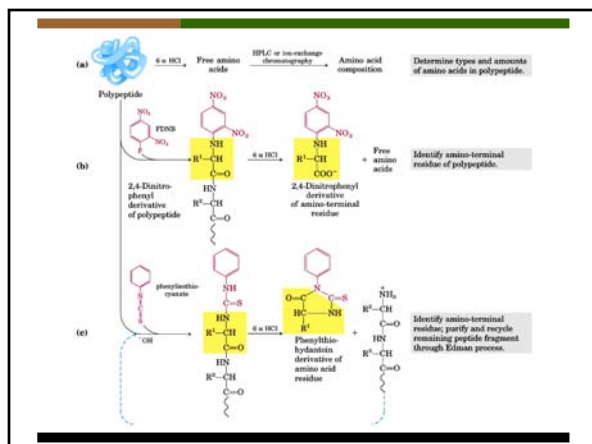


Figure 3 Amino Acid Analysis Standard Chromatogram

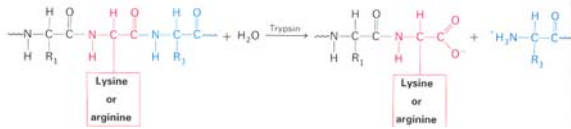


## Edman Degradation

- Sequentially removes one residue at a time from the amino end of a peptide
- Phenyl isothiocyanate (PITC) reacts with terminal amino groups of peptides to form phenyl thiocarbamyl (PTC) derivatives
- Under mildly acidic conditions the modified cyclic derivative of the terminal amino acid is liberated as a phenyl thiohydantoin (PTH)
- This creates a 'new' N-terminus and a peptide shortened by one amino acid



Proteins can be specifically cleaved into peptides to facilitate analysis



- Trypsin cleaves polypeptides on the carboxyl side of arginine and lysine residues.
- The peptides obtained after cleavage can be separated by chromatography
- It is sometimes necessary to break large peptides down further using other proteolytic enzymes such as chymotrypsin, pepsin, or papain.

## Proteases

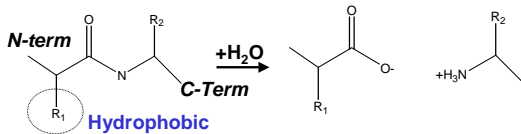
- To maintain protein turnover;
- To digest diet proteins;
- To regulate certain enzyme activities
- General hydrolysis reaction:
- A class of proteases whose catalytic mechanism is based on an active-site serine residue – serine proteases
- Include trypsin, chymotrypsin, elastase, thrombin, subtilisin, plasmin, tissue plasminogen activator etc.

## SERINE PROTEASES

**Chymotrypsin** – Needed for protein digestion.

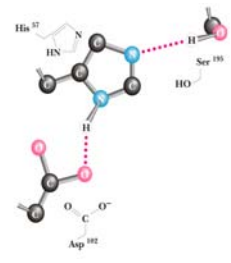
- selectively cleaves at the carboxyl side of **BULKY, HYDROPHOBIC** amino acids.

- Tyr
  - Trp
  - Phe
  - Met
- Aromatic Side Chains
- Large, hydrophobic side chain

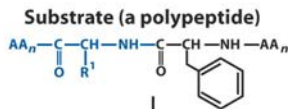


## Catalytic Mechanism of Serine Proteases

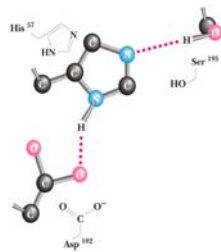
- Chymotrypsin cleaves after hydrophobic aromatic residues (Tyr, Phe, Trp, sometimes Met);
- The active site of chymotrypsin contains three conserved residues: His<sup>57</sup>, Asp<sup>102</sup>, and Ser<sup>195</sup>;
- catalytic strategy: covalent intermediate.



## Catalytic Mechanism of Serine Proteases



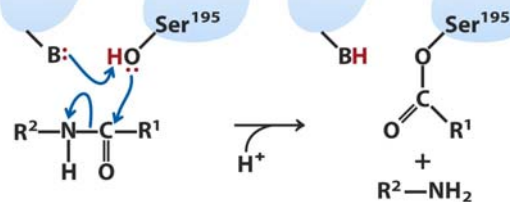
When substrate binds, the side chain of the residue adjacent to the peptide bond to be cleaved nestles in a hydrophobic pocket on the enzyme, positioning the peptide bond for attack.



Catalytic triad  
His<sup>57</sup>, Asp<sup>102</sup>, and Ser<sup>195</sup>

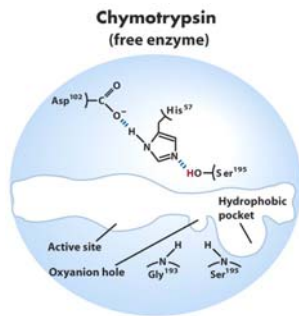
## Catalytic Mechanism of Serine Proteases

### Chymotrypsin



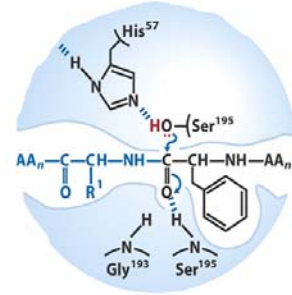
### Catalytic Mechanism of Serine Proteases

- Asp<sup>102</sup> functions only to orient His<sup>57</sup>
- His<sup>57</sup> acts as a general acid and base
- Ser<sup>195</sup> forms a covalent bond with peptide to be cleaved
- Covalent bond formation turns a trigonal C into a tetrahedral C
- The tetrahedral oxyanion intermediate is stabilized by NHs of Gly<sup>193</sup> and Ser<sup>195</sup>



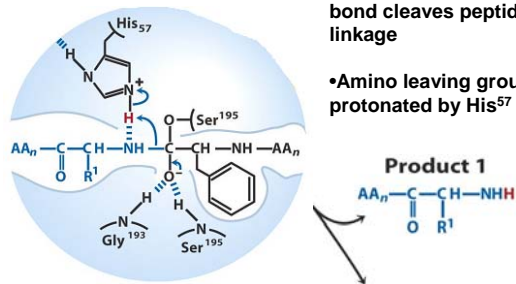
### Catalytic Mechanism of Serine Proteases

- Strong nucleophilic alkoxide ion generated on Ser<sup>195</sup> by interaction with His<sup>57</sup>
- The ion attacks the peptide carbonyl group - tetrahedral acyl enzyme formed
- Negative charge on carbonyl oxygen of substrate stabilized by H bonding in oxyanion hole

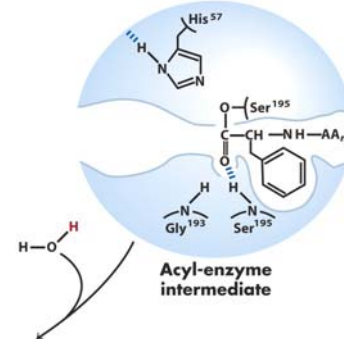


### Catalytic Mechanism of Serine Proteases

- Reformation of double bond cleaves peptide linkage
- Amino leaving group protonated by His<sup>57</sup>

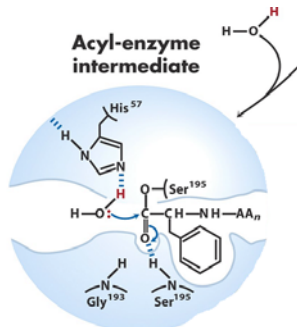


### Catalytic Mechanism of Serine Proteases



### Catalytic Mechanism of Serine Proteases

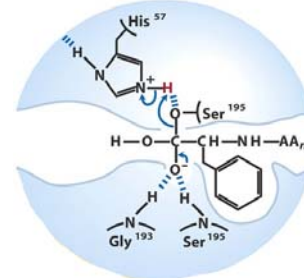
- Water molecule deprotonated generating strong nucleophilic hydroxide ion
- Attack on ester linkage – tetrahedral intermediate formed
- Oxygen in oxyanion hole takes on a negative charge



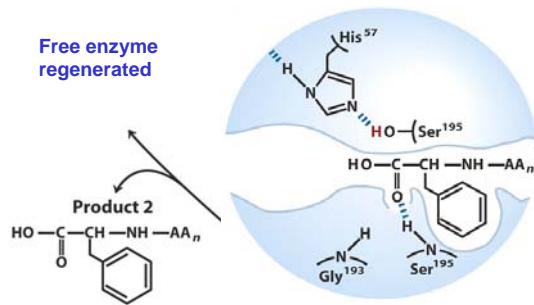
### Catalytic Mechanism of Serine Proteases

#### Deacylation

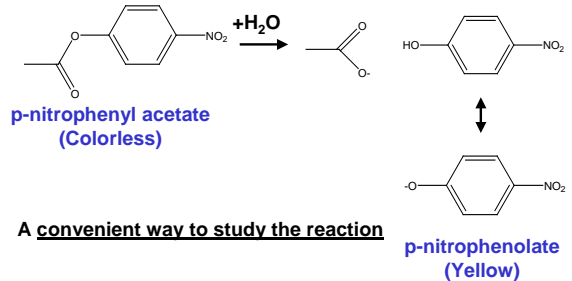
- Tetrahedral intermediate collapses forming a carboxylate ion
- Ser<sup>195</sup> displaced



### Catalytic Mechanism of Serine Proteases



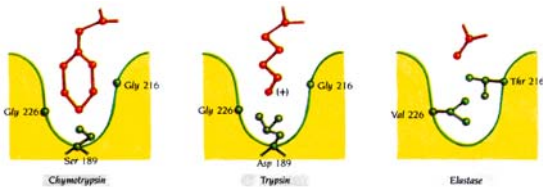
Chymotrypsin also cleaves ESTER linkages.



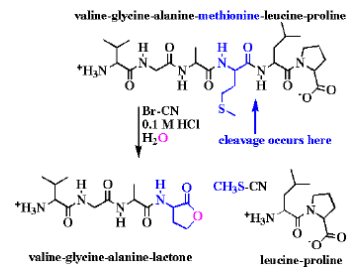
### Other Serine Proteases

- Chymotrypsin (Cuts next to Hydrophobic Groups)
- Trypsin (Cuts next to Arg & Lys)
- Elastase (Cuts next to Val & Thr)

#### Enzyme active site



**Cyanogen bromide:** reacts specifically with methionine producing peptides with C-terminal homoserine lactone residues and a new N-terminal residue.



•**Cyanogen bromide:** reacts specifically with methionine producing peptides with C-terminal homoserine lactone residues and a new N-terminal residue.

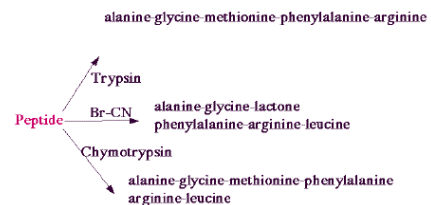
•**Trypsin** : a protease which specifically catalyzes hydrolysis of peptide bonds on the carboxyl side of lysine and arginine residues.

•**Pepsin:** preferentially cleaves at the carboxyl side of Phe, Tyr, Trp

•**Carboxypeptidase** : catalyzes cleavage of peptide bonds at the C terminus

•**Chymotrypsin** : less specific protease; preferentially catalyzes hydrolysis of peptide bonds on carboxyl side of phenylalanine, tyrosine and tryptophan

•**Thermolysin:** following amino acid is Ile, Met, Phe, Trp, Tyr, Val but not after Pro



Treatment of a sample of the intact peptide with dansyl chloride followed by acid hydrolysis produces the fluorescent sulfonamide **dansyl-Ser**.

Treatments with chymotrypsin and trypsin produced four peptides each.

**Chymotrypsin Treatment**

Ser-Ala-Arg-Gly-Pro-Leu-Trp

Ser-Phe

Val-Iso-Leu-Trp

Lys-Ser-Tyr

Glu-Gln-Lys

**Trypsin Treatment**

Ser-Tyr-Val-Iso-Leu-Trp-Glu-Gln-Lys

Gly-Pro-Leu-Trp-Lys

Ser-Phe-Ser-Ala-Arg

What is the sequence of the polypeptide?

**Ser-Phe-Ser-Ala-Arg-Gly-Pro-Leu-Trp-Lys-Ser-Tyr-Val-Iso-Leu-Trp-Glu-Gln-Lys**

1. Treatment of a sample of the peptide with 6N HCl at 120°C for 24 hours yields the following relative amounts of the various amino acids.  
3 Val 2 Leu 1 Arg 2 Met 2 Lys 1 Gly 1 Phe 2 Glu 1 Ser 1 Tyr 2 Ala 1 Trp
2. Treatment of a sample of the intact peptide with dansyl chloride followed by acid hydrolysis produces the fluorescent sulfonamide dansyl-alanine.
3. Two separate samples of the original polypeptide were treated with:  
Trypsin: yielding 3 peptide fragments  
a) met-gly-phe-glu-lys  
b) ala-val-leu-trp-arg  
c) ser-gln-met-val-tyr-ala-val-leu-lys  
Chymotrypsin: yielding 4 peptide fragments  
a) arg-met-gly-phe  
b) ala-val-leu-lys  
c) ala-val-leu-trp  
d) glu-lys-ser-gln-met-val-tyr  
Explain why there is a gln present in the tryptic peptide fragments and not one in the acid hydrolysis in 1.
4. What is the sequence of this peptide?

Ala-val-leu-trp-arg-met-gly-phe-glu-lys-ser-gln-met-val-tyr-ala-val-leu-lys

**A heptapeptide (2 M, D, R, K, F, G) was isolated and subjected to the following reactions:**

Reaction of the heptapeptide with FDNB gave DNP-M.

Limited proteolysis with carboxypeptidase indicated that M was the first amino acid released

Cyanogen bromide (CNBr) reaction with the heptapeptide released one equivalent of free homoserine lactone

Chymotrypsin digestion of the heptapeptide yielded a pentapeptide and a dipeptide. Reaction of the pentapeptide with dansyl-Cl gave dansyl-M.

Trypsin digestion yielded two M-containing tripeptides and free R.

Digestion of the heptapeptide with pepsin gave a tetrapeptide (containing M, R, K and D) and a tripeptide (M, F, and G).

**Determine the sequence of a linear, unbranched decapeptide for which the following experiments have been done:**

- Amino acid analysis gives one each of the following 10 amino acids: Trp, Gln, Met, Arg, Lys, Cys, Phe, Pro, Thr, Ala.
- Cyanogen bromide (CNBr) cleavage gives two pentapeptides.
- Trypsin cleavage gives two tripeptides (AMR and QFK), and a tetrapeptide.
- Chymotrypsin cleavage gives a dipeptide QF, a single amino acid, and a heptapeptide.
- The C-terminal residue is identified as Cys using carboxypeptidase C, which removes amino acids non-specifically, one by one, from the C-terminal end of a polypeptide.

**Determine the sequence of the following heptapeptide:**

Amino acid analysis of the heptapeptide revealed that the original peptide was composed of: R, V, Y, E, K, A and G.

Reaction of the heptapeptide with dansyl-Cl and acid hydrolysis gave dansyl-A.

Digestion of the heptapeptide with:

carboxypeptidase gave G as the first detectable amino acid.

trypsin gave free R,

a dipeptide (A-K) and a tetrapeptide containing E, G, Y and V

digestion of the tetrapeptide above (derived from the trypsin digestion above)

with chymotrypsin gave two dipeptides: V-Y and E-G.

pepsin gave a tetrapeptide and a tripeptide (Y-E-G).