# PRACTICAL 3: DIGESTIVE ENZYMES, SPECIFICITY AND pH

## 3.1 Introduction

The **aims** of this practical are:

- to illustrate the different pH dependence of gastric and pancreatic digestive proteases
- to illustrate the different substrate specificities of trypsin and chymotrypsin
- to introduce the concept of a chromogenic reaction - a reaction that produces an easily quantifiable coloured product. Such reactions are the basis of the majority of simple clinical tests you may perform yourself in the future and so it is important that you should have a feel for how they work.
- to provide an opportunity to practice handling simple numerical values (amounts and concentrations) in a laboratory situation.

## 3.2 Theory and background

***Proteins, enzymes and proteases***

Proteins are large polymers of amino acids joined by **peptide** bonds. The peptide bond is an amide linkage joining the amino group of one amino acid to the carboxyl group of another (**Fig. 1**). There are 20 different amino acids found in proteins. They differ in the chemical nature of the "side chain", **R**. When several amino acids are joined in this way, the result is a **peptide**. When large numbers (e.g. 100's) are joined together, the product is called a **polypeptide**. Proteins consist of one or more polypeptide chains held together, usually by non-covalent interactions. All polypeptides have an **N-terminus** (where the amino group of the end amino acid is unlinked) and a **C-terminus** (where the carboxyl group of the end amino acid is unlinked) (**Fig. 1**). **Enzymes** are biological catalysts that carry out all the reactions within a cell. Virtually all enzymes are proteins and the molecules upon which they act are known as their **substrates**.

**Proteases** are **proteolytic enzymes** i.e. enzymes that break the peptide bonds in other proteins and polypeptides to generate smaller peptide fragments and individual amino acids. They have numerous functions, one of which is to generate amino acids from protein in food for reutilisation. **Pepsin** is a protease that is secreted from the gastric mucosa into the stomach where the conditions are very acidic due to the presence of HCl in gastric secretions. Thus pepsin must be able to operate at very **low pH** (high acidity), something that is unusual for an enzyme. Once the gastric contents pass into the duodenum, the pH is increased above 7 into the slightly alkaline

region by bicarbonate in the pancreatic secretions. These secretions also contain proteases such as **trypsin**, **chymotrypsin** and **elastase** which break down dietary proteins further. These proteases must be able to operate at alkaline pH.

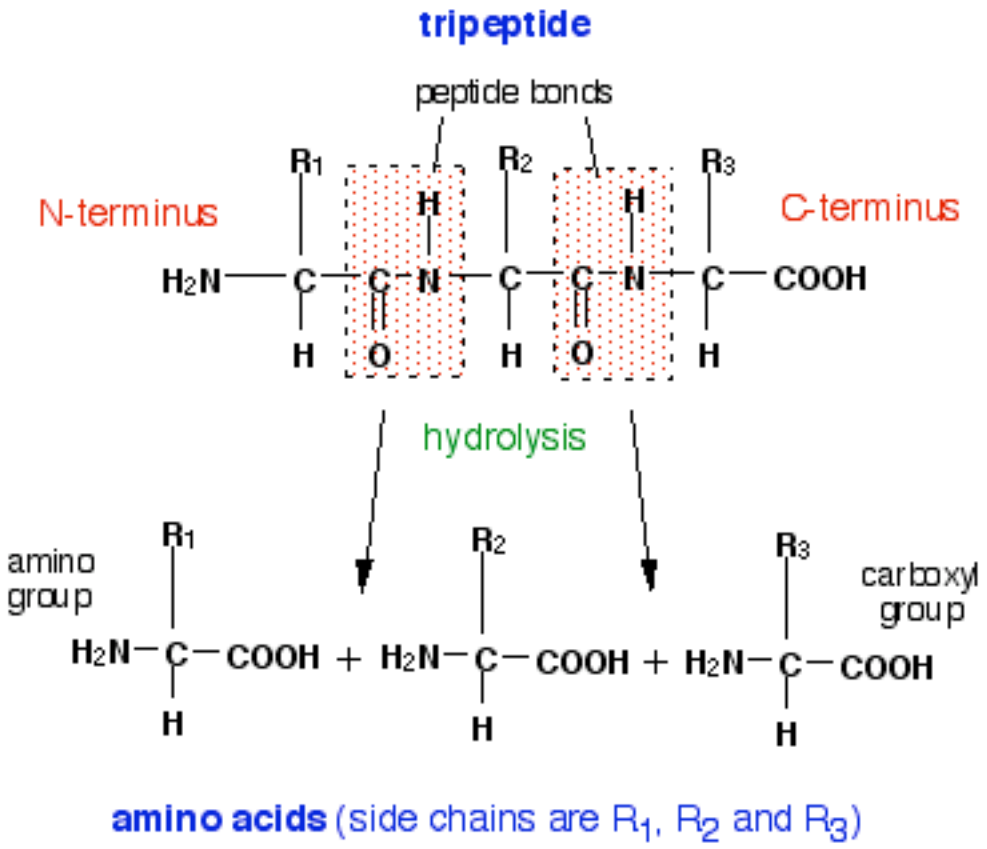


Figure 1: Peptides, peptide bonds and amino acids

- *In experiments A, B and D, you will determine the activities of trypsin, chymotrypsin and pepsin across the pH range 1 to 10 to illustrate their different pH dependencies.*

***Substrate specificity of proteases***

Some proteases (**exopeptidases**) start at the end of a polypeptide chain and sequentially remove one amino acid at a time. Others (**endopeptidases**) break internal peptide bonds and initially produce peptide fragments. Trypsin, chymotrypsin and pepsin are all endopeptidases. Some endopeptidases are relatively non-specific and **hydrolyse** the peptide bond between any two of the 20 amino acids while others have a strong or absolute preference for certain amino acids. Thus, trypsin only cleaves the peptide bonds after (on the C-terminal side of) the basic amino

acids **lysine** and **arginine** while chymotrypsin prefers cleaving after large **hydrophobic** amino acids such as **phenylalanine**, **tyrosine** and **tryptophan**, and also **leucine** and **methionine** (**Fig. 2**). This difference in specificity depends on the nature of the amino acid side chains present in the **active site** of the proteases that bind to their substrates. **Pepsin** is relatively non-specific although it does have a preference for cleaving after hydrophobic amino acids. In digestion, a combination of exopeptidases and endopeptidases with different substrate specificities will reduce most dietary proteins to individual amino acids.

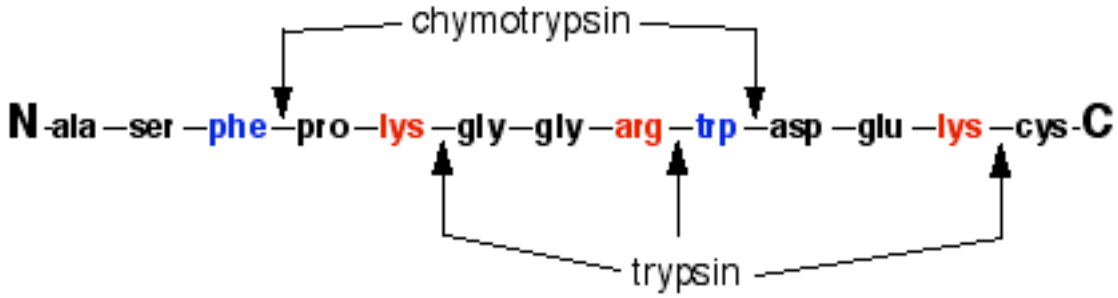


Figure 2: Peptide bond cleavage specificity of trypsin and chymotrypsin.

- *In experiment C, you will demonstrate the different substrate specificities of trypsin and chymotrypsin.*

***Chromogenic reactions and colorimetry***

"**Chromogenic**" means "producing colour". The intensity (or **absorbance**) of a solution of a coloured compound is directly proportional to the concentration of that compound. Thus, if a chemical or biochemical reaction produces a coloured product from colourless reactants, then the rate and extent of the reaction is very easily measured by measuring the absorbance of the reaction after a given time.

Absorbance is most simply measured in an instrument called a **colorimeter**. This simply consists of a white light source and a light detector and the coloured solution is placed between these to see how much light is absorbed by it. Sensitivity is increased by providing the range of colour wavelengths that the solution is known to absorb, e.g. **red** solutions absorb **blue** light (that's why they look red), so by placing a blue filter after the light source only blue light is provided to the solution, so a greater percentage of this **incident light** will be absorbed, thus increasing the sensitivity of detection. Greatest sensitivity and specificity is provided by **spectrophotometers**, which deliver single wavelengths of light (e.g. 410 nm or 650 nm) rather than wavelength ranges (blue, green etc).

Unfortunately, most reactions we would like to measure do not produce coloured products directly, so we have to adapt them. There are three main ways of doing this, and you will use two of these in this practical and see an example of the third in a later practical..

a) The first is to replace the natural substrate with a synthetic one that will generate a colour when it undergoes the reaction. **N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA)** is a simple colourless ester which is recognised by trypsin as a substrate (trypsin is an **esterase** as well as a protease). Trypsin cleaves the bond between the arginine (remember the specificity of trypsin from above) and the p-nitroaniline to release free **p-nitroaniline**, which is **yellow** and easily measured in a colorimeter. Because of its different substrate specificity, chymotrypsin will not cleave BAPNA. On the other hand, chymotrypsin will cleave **N-succinyl-L-phenylalanine p-nitroanilide (NSLPN)** as it has a large hydrophobic amino acid (phenylalanine) in the correct position. As expected, trypsin does not cleave NSLPN so these two simple compounds can be used to demonstrate the different substrate specificities of these two proteases as well as being useful for measuring their **pH optima** (Experiments A, B and C).

b) The second method is to treat the colourless product with a chemical that then produces a colour, i.e. if compound **AB** breaks down to **A** + **B** (both colourless), but **B** reacts with **C** to produce **D** (coloured), then the amount of **D** can be used to indicate the amount of **B** produced. **Ninhydrin** is a compound that reacts with amines to produce an intense blue-purple colour. All proteins have a single free amino group at their N-terminus, but when they are hydrolysed the total number of free amino groups increases as peptides and amino acids are produced. You will use the ninhydrin reaction to measure the hydrolysis of the protein **haemoglobin** by pepsin at different pH values (Experiment D).

(*Technical note*: In addition to the free N-terminal amino group, most proteins have free amino groups on the side chain of the amino acid **lysine**. These also react with ninhydrin and can produce a high background colour. Hence, the haemoglobin substrate used has been pre-treated with formaldehyde to chemically block these extra amino groups. The slight blue background you will still see is due to the pepsin, which is of course a protein. It cannot be pre-treated as this would destroy its enzyme activity).

c) The third method is to couple the first reaction to a second (and sometimes third), which finally produces a coloured product (more later).

## 3.3 Experiment A: Construction of calibration curve

**Reagents:** **1 mM *p*-nitroaniline (p-NA)**
**Distilled water**

This calibration curve relates the intensity of the yellow colour of *p*-nitroaniline at 430 nm to its concentration. It will be used to analyse the data in Experiments B, C and D.

**Procedure:**

1. Label six test tubes with a magic marker, then, using the appropriate automatic pipette (see **Appendix 2** for instructions), pipette either 2.0 ml, 1.6 ml, 1.2 ml, 0.8 ml, 0.4 ml or 0 ml of the yellow **1 mM *p*-nitroaniline (p-NA)** standard into each tube in a test tube rack. In each case make up the total volume to 2.0 ml with the appropriate amount of water to give six calibration standards. **Check that all tubes have the same final volume by eye: if not, you've made a pipetting error.** Calculate the p-NA concentration (mM) **AND** the number of micromoles (μmol) in each tube then measure the absorbance of each in the colorimeter with the 430 nm filter using a water blank (see **Appendix 3** for instructions). Complete the table below then plot absorbance (*y*-axis) against the number of μmol *p*-NA (*x*-axis), labelling the graph appropriately.

| Volume (ml) | 2.0 | 1.6 | 1.2 | 0.8 | 0.4 | 0 |
|---|---|---|---|---|---|---|
| Concentration (mM) | | | | | | |
| No. of μmol per tube | | | | | | |
| Absorbance | | | | | | |

## 3.4 Experiment B: Determination of the pH optimum of trypsin

**Reagents:** **2 mM N-□-benzoyl-DL-arginine-p-nitroanilide (BAPNA)**
**0.1 mg/ml trypsin** (in 20 mM $CaCl_2$) ***KEEP THIS STOCK ON ICE***
**0.2 M various assay buffers, pH range 1 to 10**

**Procedure:**

1. (11 tubes in all). Prepare 10 **labeled** assay tubes as follows: Pipette 1.0 ml of each of the 10 assay buffers (from pH 1 to 10) into 10 separate test tubes then add 0.9 ml of the **enzyme substrate**, **2 mM BAPNA** to each and mix well. To an 11$^{th}$ **control** tube, add 1.1 ml water and 0.9 ml 2 mM BAPNA. Then, quickly but carefully, add 0.1 ml trypsin to each of tubes 1 to 10 (but **NOT** tube 11) and **mix well**. **Note that thorough mixing of the contents of**

**the tubes is essential**. **Again, check that all tubes appear to have the same final volume.** Finally, place all 11 tubes in the 37˚ water bath for 15 min.

2. After 15 min incubation, measure the absorbance of each of the 10 assay tubes at 430 nm using the control tube as a blank. Do this quickly but carefully so that all tubes will have been incubated for about the same time — 15 min. Using the calibration curve, convert the absorbance values into µmol p-NA and complete the table below. Plot enzyme activity (µmol p-NA produced per min, *y*-axis) against pH (*x*-axis).

| pH | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Absorbance | | | | | | | | | | |
| µmol p-NA/min | | | | | | | | | | |

## 3.5 Experiment C: Determination of the pH optimum of chymotrypsin

**Reagents:** **2 mM N-succinyl-L-phenylalanine p-nitroanilide (NSLPN)**
**5 mg/ml α-chymotrypsin** (in 20 mM $CaCl_2$) ***KEEP THIS STOCK ON ICE***
**0.2 M various assay buffers, pH range 1 to 10**

**Procedure:**

Repeat the procedure exactly as described for trypsin in **3.4** substituting **NSLPN** as substrate and **chymotrypsin** as enzyme. Calculate the data as before and plot on the **SAME** piece of graph paper as the trypsin curve using a different symbol for the chymotrypsin points.

| pH | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Absorbance | | | | | | | | | | |
| µmol p-NA/min | | | | | | | | | | |

## 3.6 Experiment D: Determination of the substrate specificity of trypsin and chymotrypsin

**Reagents:** **As above**

**Procedure:**

Prepare a set of four **labelled** tubes containing all combinations of 0.1 ml **trypsin** or **chymotrypsin** with 0.9 ml **BAPNA** or **NSLPN** according to the table below. Use 1.0 ml pH 8 buffer in all four tubes. Add the enzyme LAST, **mix well**, then incubate at 37˚ for 15 min. Measure the absorbance at 430 nm using a water blank, calculate the amount of product in each case and enter the values into the table.

| | Trypsin | Chymo-trypsin | BAPNA | NSLPN | Absorbance | µmol product/min |
|---|---|---|---|---|---|---|
| 1 | + | - | + | - | | |
| 2 | + | - | - | + | | |
| 3 | - | + | + | - | | |
| 4 | - | + | - | + | | |

## 3.7 Experiment E: Determination of pH optimum of pepsin

**Reagents:** **0.2 % (w/v) N,N-dimethylated haemoglobin (Hb)** (in 10 mM NaCl)
**1 mg/ml pepsin** (in 10 mM NaCl)
**0.2 M various assay buffers**
**stop solution** (0.2 M Tris-HCl, pH 8.5)
**ninhydrin reagent**

**Procedure: (<u>12</u> tubes in all)**

1. Prepare 10 **labelled** assay tubes as follows: Pipette 0.20 ml of each of the 10 assay buffers (from pH 1 to 10) into 10 separate test tubes then add 0.25 ml of the **enzyme substrate**, **0.2 % Hb,** to each and mix well. Then, quickly but carefully, add 0.05 ml (50 µl) pepsin solution to each of the tubes, mix well, and place all tubes in the 37˚ water bath for 30 min.

2. Prepare two control tubes as follows: to one (tube 11), add 0.45 ml water and 0.05 ml pepsin and to the other (tube 12) add 0.25 ml water and 0.25 ml 0.2% Hb. Incubate these with the other tubes.

3. N.B. FROM THIS POINT ON WEAR DISPOSABLE GLOVES AS THE NINHYDRIN REAGENT WILL STAIN YOUR HANDS BRIGHT BLUE!

4. After 30 min, remove the tubes from the water bath and add 0.2 ml "**stop solution**".

5. Place in the 80˚ water bath for 5min, then remove.

6. Next, add 1.0 ml **ninhydrin** reagent. Mix **carefully** by tapping the sides of the tubes (don't spill any) then place in the 80˚ water bath for a further 10 min to develop the colour.

7. Remove the tubes, allow to cool, then observe the colour that has developed.

You should notice that the tubes in which pepsin is inactive contain a precipitate of undigested haemoglobin that has been denatured by heating at 80˚. In order to measure the absorbance of the blue colour in the colorimeter, you would need to remove this precipitate by centrifugation. Instead, you will simply score the absorbance visually using the scale (+++) – strong blue, (++) – medium blue, (+) – light blue, (-) – no colour. Score the "volume" of precipitate in the same manner as it gives a complementary indication of digestion.

| pH/tube | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Blue colour | | | | | | | | | | | | |
| Precipitate | | | | | | | | | | | | |

## 3.8 Conclusions:

For future reference, you should note your conclusions here. Some of these can be worked out directly from the results, others will require some literature searching. Examples of what you should be thinking about are:

- Over what pH range are trypsin and chymotrypsin active and does this reflect their working environment *in vivo*?

- What is the molecular basis for the demonstrated substrate specificity of trypsin and chymotrypsin. What might the relevance of this be *in vivo*?

- Over what pH range is pepsin active and does this reflect its working environment *in vivo*?

- Is the digestion of protein in the stomach by pepsin directly important for protein breakdown *per se* or might there be some other purpose/benefit in the production of gastric amino acids and peptides?

- Why do the proteases stored in the gastric mucosa and pancreas not digest these tissues?

- Why do proteins aggregate and precipitate when heated in solution?

- Assuming that not all the haemoglobin in the tubes where pepsin is **active** has been completely digested to amino acids (true), why is there no precipitate of undigested Hb in these tubes?

**APPPENDIX 2:**

**Use of automatic pipettes**

You will make extensive use of automatic pipettes with disposable tips in these practicals. Please treat these with care. Do not drop them. There are three different pipettes which are used for different volume ranges. Make sure you select the correct pipette and tip (**yellow** tips for orange and yellow button pipettes; **blue** tips for blue button pipettes)

| | Volume range (µl) | Volume range (ml) |
|---|---|---|
| Orange button | 5 – 40 | 0.005 – 0.04 |
| Yellow button | 40 – 200 | 0.04 – 0.2 |
| Blue button | 200 – 1000 | 0.2 – 1.0 |

1. Firmly push the correct tip on to the end of the pipette.

2. Dial up the required volume (in **microlitres**) by twisting the coloured button. **N.B. Do not try to dial in a figure above or below the stated volume range or you will jam the pipette. If you need a volume outside the stated range, choose a different pipette.**

3. Holding the pipette by the barrel, push down the button with your thumb to the **first** position of resistance and hold it there.

4. Submerge the disposable tip just below the surface of the liquid to be pipetted and **gently** allow the plunger to rise again by **slowly** releasing the pressure from your thumb (do **not** just take your thumb off the end and allow the plunger to shoot up otherwise you will get liquid into the barrel of the pipette). The correct volume of liquid should now be in the tip. Once the plunger has risen fully (and **NOT** before), remove the pipette tip from the liquid. Check that the volume looks right (this becomes easier with experience) and that there are no air bubbles or air gaps.

5. Place the tip in the receiving tube (near the bottom and touching against the side) and slowly push down on the plunger with your thumb through the first position of resistance to the second. Remove the pipette from the tube and check that all the liquid is out of the tip.

6. You can continue to use the same tip if (a) you are pipetting more of the same solution or (b) if you are pipetting dilutions of the same solution as long as you are working from the most dilute to the most concentrated (but **NOT** the other way round). Otherwise eject the tip into a waste bin by pushing down on the eject slider with your thumb.

**APPENDIX 3:**

**Use of colorimeters**

There are two different types of instrument in the laboratory (Griffin and Jenway). The procedures are similar, but make sure you know which type of instrument you are using and always use the same machine for your measurements.

The filters are used to isolate a part of the visible light spectrum that is absorbed maximally by the sample. Different colorimeters use different sets of filters but typical wavelengths passed are red filter: 630-750nm, green filter: 510-570nm and blue filter: 360-480nm. Although you will normally be told which filter to use you should consider and understand the reason for this choice.

**Operating instructions for colorimeter**

1. Switch on the instrument at least 5 minutes before use to allow it to stabilize.

2. Select the most appropriate filter for the analysis and insert it in the light path (Griffin) or dial it in with the selector (Jenway).

3. Place the reagent blank solution (or water) in the cuvette and zero the instrument (either using the 'set zero' control (Jenway) or the 'calibrate' switch (Griffin). Make sure the clear faces of the cuvette are in the light path (which runs from back to front in the Jenway but side to side in the Griffin).

4. Place the sample in the colorimeter and read the absorbance of the solution. If the absorbance is "over range" (usually > 2.0) then the sample must be diluted to yield a value within the limits of the instrument.

5. At intervals, recheck the reagent blank to ensure that there is no drift in the zero value.

**Notes**

- The machine must be re-zeroed (step 3) if a new filter is chosen.

- The sample cuvettes must be at least two-thirds full.

- The outside of the cuvette must be dry and clean.

- If an instrument appears to be faulty, do not abandon it but consult a demonstrator!