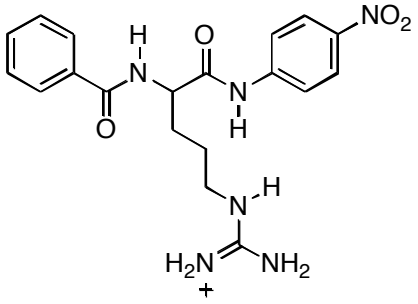
Determination of the pH Optimum of Trypsin

Every enzyme has a pH optimum at which it catalyzes reactions most efficiently. Trypsin is a serine protease that cleaves peptide bonds on the C-side of lysine and arginine. The rates of Trypsin catalyzed peptide bond hydrolysis reactions can be evaluated *in vitro* by using artificial substrates that generate chromophoric products. For example, hydrolysis of the substrate N-benzoyl-DL-arginine p-nitroanilide produces p-nitroanaline (BAPNA), a molecule that absorbs light at 410 nm. In this experiment the pH optimum of Trypsin will be determined by carrying out the hydrolysis of BAPNA at various values of pH and plotting the enzyme activity (μmoles of p-nitroaniline produced per minute per unit weight of enzyme) against this parameter.



BAPNA

Reagents: (Please estimate the volumes of the reagents you will need for this experiment and use the minimum amount necessary.)
1.0 mM p-nitroaniline (p-NA, MW 138)
0.3 mM N-benzoyl-DL-arginine p-nitroanilide (BAPNA, MW 435)
0.1 mg/mL trypsin in 20 mM $CaCl_2$ (This solution is kept on ice.)
0.2 M phosphate buffer solutions ranging in pH from 5.0 to 10.0 in increments of 1 pH unit

Experimental:
Preparation of a standard curve: Transfer 0.1, 0.2, 0.3, 0.4 and 0.8 mL of 1.0 mM p-NA into each of five test tubes and dilute each of the samples to a total volume of 5.0 mL with deionized water. A sixth tube containing only deionized water will serve as a blank. Measure the absorbance of each solution at 410 nm. Calculate the concentration of p-NA in each tube in units of mM and the number of μmoles of p-NA in each sample. Construct a plot of A_{410} against μmoles of p-NA.

Rate vs. pH Profile: Transfer 1.0 mL of the assay buffers (pH 5.0 to pH 10.0), 3.0 mL of deionized water and 0.9 mL of 0.3 mM BAPNA (the substrate) into each of six test tubes. (The tubes now contain 0.27 μmol of BAPNA, each at a different pH.) Add 0.9 mL 2.0 mM BAPNA and 4.1 mL of deionized water to a seventh tube (the control). Then transfer 0.1 mL of the Trypsin solution to each of the first six tubes, mix and incubate at 37^{O}C for 15 minutes. Start the incubation periods at one-minute intervals beginning with the blank. After 15 minutes of incubation, measure the absorbance of each sample using the control tube as a blank. Six separate measurements will be made at one-minute intervals. Set up a Table in your notebook that has the Volumes of buffer, BAPNA, water and Trypsin solution, the pH and the measured absorbance after the 15-minute incubation period.

- Determine the pH optimum by plotting rate (μmoles of p-NA/min) versus pH
- Calculate the specific activity (μmoles of p-NA/min per mg protein) at the pH optimum
- Calculate the number of molecules of BAPNA converted to product per molecule of Trypsin per minute. Trypsin has a MW of 15,000 and contains a single active site
- Evaluate whether or not the reaction was carried out under saturating conditions. (Calculate the ratio of substrate to enzyme at the beginning of the reaction.)
- Calculate the percent of substrate that reacted during the incubation period
- Find the literature value of the pH optimum of Trypsin and compare it to your experimentally determined value.