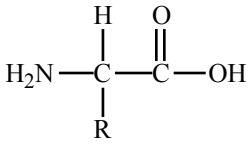
Experiment 9 – Amino Acids and Proteins

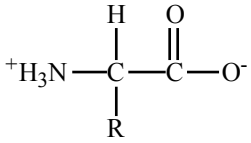
Proteins are very important biological molecules, with many possible functions. Enzymes are proteins that catalyze biological reactions. There are transport proteins (such as hemoglobin), storage proteins, structural proteins, proteins that do the work of muscular contraction, and other types of proteins. All proteins are very large molecules with huge molecular weights. Proteins are polymers of amino acids – they consist of long chains of amino acids linked together by peptide bonds.

An **amino acid** is a molecule that contains an amino group and a carboxyl group in the same molecule. The amino acids that are found in proteins are α-amino acids, which means that the amino group is attached to the alpha carbon (the carbon next to the carboxyl group). There are 20 amino acids that differ from each other only in the identity of the side chain attached to the alpha-carbon. The amino acid side chains can be classified based on whether they are nonpolar, polar, acidic, or basic. The general structure of an amino acid is shown below. In the following structure, R represents the side chain.

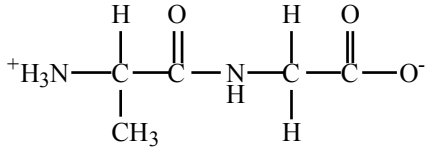


Since proteins are biological molecules, they are usually found in a neutral solution that is buffered at pH 7.0 to 7.4. In this situation, the acidic and basic groups on the amino acids will be ionized. Most amino acids exist as "**zwitterions**" (dipolar ions) at pH 7. The structure of a zwitterion is shown below. Because of the charges, amino acids are water-soluble.

Amino acids can be linked together – the amino group on one amino acid can



react with the carboxyl group on another amino acid, forming an amide. Therefore, the bonds linking together the amino acid residues are amide bonds, but in proteins they are called **peptide bonds**. Shown on the next page is the structure of a **dipeptide**, which is two amino acids linked by a peptide bond. A typical protein molecule contains around 100 amino acids joined by peptide bonds.



Alanylglycine, a dipeptide

There are many levels to the structure of a protein. The **primary structure** of a protein is the sequence of amino acids. This sequence actually determines the overall shape of the protein. The **secondary structure** consists of regular and repeating

structures held together by hydrogen bonds between the C=O and the N-H groups along the backbone of the molecule. The alpha helix and beta sheet are examples of secondary structure. The **tertiary structure** refers to the overall folding of the entire polypeptide chain, and it is determined by interactions between the side chains. These interactions include hydrogen bonding, hydrophobic interactions, salt bridges, and disulfide bridges. **Quaternary structure** involves the same types of interactions as tertiary structure, but the interactions occur between the side chains on different polypeptide chains. These interactions hold together different subunits of a protein.

If the overall folding of a protein is disrupted, we say that the protein is **denatured**. A denatured protein loses its biological activity. Some common denaturing agents include heat, organic solvents, acids, bases, agitation, detergents, and heavy metal ions. It is important to note that denaturation affects the secondary, tertiary, and quaternary structure of a protein, but doesn't affect the primary structure. Sometimes the denaturation is reversible and the protein can be renatured.

Paper Chromatography of Amino Acids

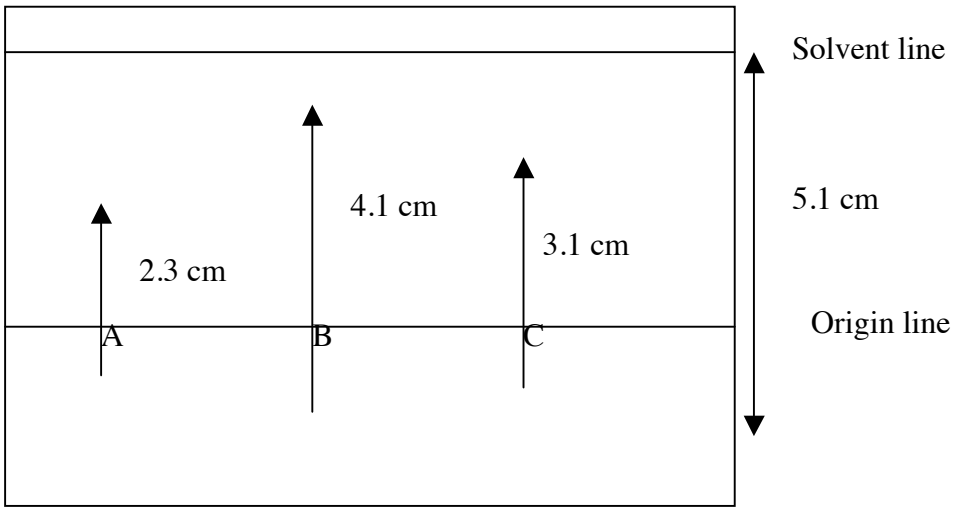
Chromatography is a technique of separation and identification. There are many types of chromatography, including paper chromatography, thin layer chromatography, gas chromatography, liquid chromatography, ion-exchange chromatography, and so on. We will be using paper chromatography to identify amino acids.

In paper chromatography, the samples to be tested are placed near one edge of a rectangular piece of filter paper. The paper is then placed in a container that has some solvent on the bottom. The solvent gradually moves up the paper, carrying the samples with it. The samples will travel at different rates: those that are more attracted to the solvent will travel faster, and those that are more attracted to the paper will move slower. After the paper is taken out and dried, it is sprayed with ninhydrin. This will color the amino acid spots purple. (Since the amino acid solutions are colorless, we can't see where they are on the paper without some kind of dye.)

After the paper is developed, the distance that the solvent traveled and the distance that each amino acid has traveled are measured. The R_f value for each amino acid is then calculated. By comparing R_f values of unknown amino acids to known values, the unknowns can be identified.

An example calculation of R_f values is shown below.

$$R_f = \frac{\textbf{distance traveled by amino acid}}{\textbf{distance traveled by solvent}}$$



Spot A $R_f = \frac{\textbf{2.3 cm}}{\textbf{5.1 cm}} = \textbf{0.45}$ **Spot C** $R_f = \frac{\textbf{3.1 cm}}{\textbf{5.1 cm}} = \textbf{0.61}$

Spot B $R_f = \frac{\textbf{4.1 cm}}{\textbf{5.1 cm}} = \textbf{0.80}$

In this experiment, you will test several known amino acid solutions and one unknown. The results for your unknown should match one of the known amino acids.

Reactions of Amino Acids and Proteins

Hydrolysis

Proteins can be hydrolyzed either partially or completely in the presence of acids, bases, or digestive enzymes. When a protein is hydrolyzed, some or all of the peptide bonds are broken. The products obtained depend on how long the hydrolysis is allowed to take place. The products of partial hydrolysis are peptides and the products of complete hydrolysis are amino acids.

Biuret Test

Compounds that contain two or more peptide bonds will react with Cu^{2+} in a basic solution to form a violet-pink complex. The original Cu^{2+} solution is blue, so if the solution remains blue, the compound being tested could be an amino acid or a dipeptide or neither.

Xanthoproteic Test

The aromatic rings on tyrosine and tryptophan react with nitric acid. In this reaction, the aromatic rings become nitrated. When nitric acid is added to a sample and the mixture is heated, a yellow solution will result if the sample contains tyrosine or tryptophan. When this yellow solution is treated with a strong base (such as NaOH), it turns orange. Since most proteins contain one or both of these amino acids, most proteins will show a positive reaction in this test.

Ninhydrin Test

Free amino groups will react with the ninhydrin reagent to yield a purple solution. Almost all amino acids contain a free amino group (except proline and hydroxyproline). Some proteins also give a positive test with ninhydrin.

Sulfur Test

Sulfur-containing amino acids include cysteine and methionine. If a sample that contains one or both of these amino acids is acidified, the gas H_2S is produced, which smells like rotten eggs. When a piece of moistened lead (II) acetate paper is held over the solution as the H_2S is being produced, the H_2S reacts with the lead ion, forming a black or gray coating of PbS (lead (II) sulfide). Appearance of this black color is taken as a positive test for a sulfur-containing amino acid.

Denaturation

There are many ways to denature a protein. Recall that denaturation refers to a disruption of the secondary, tertiary, and quaternary structures – denaturation destroys the normal folding of the protein, making it inactive. When a protein is denatured, it often coagulates, forming a visible solid. Some denaturing agents include heat, organic solvents, agitation, acid or base, and heavy metal ions.

Safety Precautions:

- Wear your safety goggles.
- Nitric acid is very corrosive – avoid skin contact with it. If skin contact occurs, flood the area with plenty of running water for 10 minutes.
- Heavy metal ions such as Pb^{2+} and Hg_2^{2+} are poisonous. Avoid skin contact with solutions containing these ions and with the lead acetate paper. Wash your hands thoroughly before leaving the lab.
- Ninhydrin spray causes stains.

Waste Disposal:

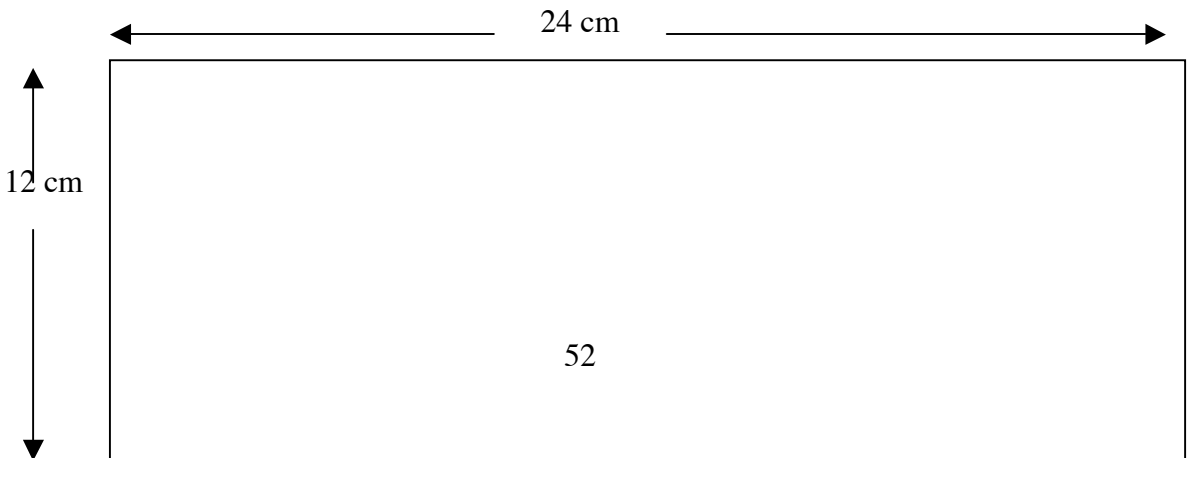
- All waste must be placed in the **inorganic** waste containers (which have a blue label) in one of the fume hoods.

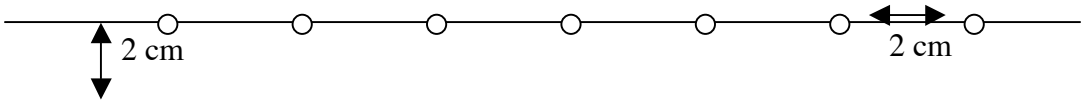
Procedure

Note: be sure to shake the protein solutions before obtaining your sample, because some of the proteins tend to settle out.

Part 1: Paper Chromatography of Amino Acids

1. Put on a pair of plastic gloves and then pick up a piece of chromatography paper that has been cut to a size of 12 cm × 24 cm. (Do not touch the paper with your fingers, because there are traces of amino acids on your skin.) Roll the paper into a cylinder and make sure that it will fit inside a large beaker (400-mL or 600-mL) without touching the sides of the beaker. If it doesn't fit, cut it to size with a pair of scissors.
2. Unroll the paper and use a pencil (not a pen!) to draw a line across the paper (the long way) so that it is 2 cm from the edge of the paper. This will be the starting point for the amino acids (this is also called the origin line). With the pencil, mark seven points along this line, making sure that they are at least 2 cm apart and at least 2 cm from either edge of the paper.





3. Using a toothpick or a capillary tube, apply a small spot of each amino acid solution to the marked spots on the paper. You will test phenylalanine, alanine, glutamic acid, serine, lysine, aspartic acid, and an unknown amino acid. Use a different applicator for each amino acid and be careful not to mix them up. (If the amino acid solutions get contaminated with traces of other amino acids, your results will be inconclusive.) Apply a small spot by lightly touching the applicator to the paper. Let the spot dry, and repeat the process on the same area to apply more amino acid. When you do this, make sure to keep the diameter of the spot as small as possible. Label each spot with a pencil as you go along. When you have applied a different amino acid solution to each of the spots, allow them to dry.
4. Take a 600-mL or 400-mL beaker to the hood. Label it with your name. Pour some of the chromatography solvent into the beaker until it reaches a depth of about 1 cm. (The liquid level must be below the origin line on your chromatography paper.) Cover the beaker tightly with plastic wrap and leave it in the hood.
5. Roll your chromatography paper into a cylinder with the spots and labels facing out. Staple the edges together, but do not let them overlap. Remove the plastic wrap from the beaker and carefully lower the cylinder into the beaker so that the row of amino acids is on the bottom edge of the paper. Do not let the paper touch the sides of the beaker. Cover the beaker with plastic wrap and leave it where it is in the hood. Do not move the beaker. Let the solvent rise until it is 2-3 cm from the top of the paper. (This may take 45-60 minutes.) Do not let the solvent reach the top of the paper. While you are waiting, go on to the other parts of the experiment.
6. When the solvent has risen to within 2-3 cm from the top of the paper, remove the paper from the beaker, remove the staples, and spread it on a paper towel to dry. **Immediately** mark the solvent line with a pencil. Let the paper dry completely.
7. While you are waiting, dispose of the solvent in the waste solvent container.
8. When the paper is completely dry, take it to the hood and spray it evenly with a small amount of ninhydrin solution. **Important: use the ninhydrin spray in the hood. Do not breathe the fumes or get the spray on your skin.** Let the paper dry. Purple spots should appear which indicate the location of the amino acids on the paper.
9. Measure the distance between the origin line and the solvent line. This distance is the distance traveled by the solvent.
10. Draw a dot in pencil at the center of each amino acid spot. Measure the distance between the origin line and the center of each spot. This is the distance traveled by the amino acid.
11. Calculate the R_f value for each of the spots.

$$R_f = \frac{\textbf{distance traveled by amino acid}}{\textbf{distance traveled by solvent}}$$

Determine the identity of the unknown by comparing its R_f value and color to those of the other spots.

Part 2: pH Test

12. You will test solutions of glutamic acid, lysine, and serine. One by one, stir the amino acid sample with a glass stirring rod and then touch it to a piece of pH paper (not litmus paper). Note and record the pH (by comparing the color of the spot to the pH scale on the box of pH paper -be sure to look at both sides of the box). Make sure to rinse and wipe off the stirring rod between solutions so that you don't accidentally contaminate them.

Part 3: Biuret Test

13. In this part, you will test solutions of egg albumin, gelatin, casein, glycine, and proline. Put 2 mL of each solution to be tested in its own test tube. Add 3 mL of 10% NaOH to each tube and mix well. (Remember, if you are using a stirring rod, rinse it and wipe it off between solutions so that you don't contaminate the solutions.) Add 2 drops of 2% copper sulfate ($CuSO_4$) solution (which is blue) to each tube and mix well. Record your observations.

Part 4: Xanthoproteic Test

14. You will test solutions of egg albumin and tyrosine. Put about 1 mL of each solution to be tested in its own test tube. In the hood, carefully add 5 drops of concentrated nitric acid (HNO_3). **Caution: the nitric acid must be kept under the hood at all times. Avoid all skin contact with nitric acid.** Mix each test tube carefully and place the tubes in a boiling water bath for two minutes. Observe and record the color of the solutions. Remove them from the water bath and let them cool. When they are cool, add 10% NaOH to each tube dropwise until the solutions are basic. (You can do this by adding one drop of NaOH, mixing well with a stirring rod, and touching the stirring rod to a piece of red litmus paper. If the litmus paper does not turn blue, add another drop of NaOH to the solution, mix with the stirring rod, and again touch it to a piece of red litmus paper. Repeat this sequence until the litmus paper turns blue – then your solution is basic. You can use the same piece of litmus paper, as long as you touch the stirring rod to a different dry section each time.) Observe and record any color changes.

Part 5: Ninhydrin Test

15. You will test solutions of glycine, proline, and egg albumin. Put 2 mL of each solution to be tested in separate test tubes. Add 1 mL of 1 % ninhydrin solution to each tube. Heat the tubes for several minutes in a boiling water bath, and record your observations.

Part 6: Sulfur Test

16. For this part, you will test solid egg albumin and solid cysteine. Weigh out about 0.2 g of each of these solids and place the samples in separate test tubes. Add 10 mL of 3 M NaOH to each tube, and place the tubes in a boiling water bath for 15 minutes. If the solutions start to foam, remove them from the water bath briefly. After 15 minutes of heating, remove the tubes from the water bath and let them cool to room temperature. Add about 10 mL of 3 M HCl to each tube to acidify the contents. Moisten two pieces of lead acetate paper with deionized water and place a piece over the top of each tube. Put the tubes in the boiling water bath again. Do not let the lead acetate paper fall off. Record your observations. **Wash you hands after handling the lead acetate paper, as lead compounds are toxic.**

Part 7: Denaturation Tests

17. Put 3 mL of egg albumin solution in a test tube and place the test tube in a boiling water bath. Observe the appearance of the solution before and after heating.

18. Put 3 mL of egg albumin solution in a test tube and add 7 mL of 95 % ethanol. Mix well and record your observations.

19. Put 3 mL of egg albumin solution in a test tube and add 5-8 drops of $FeCl_3$ solution. Mix well and record your observations.

20. Put 3 mL of egg albumin solution in a test tube and add 5-8 drops of 0.2 M Hg_2Cl_2 solution. Mix well and record your observations. **Caution: mercury compounds are toxic. Avoid skin contact with the mercury solution and be sure to put the waste in the inorganic waste container (blue label) in the hood.**

Questions

1. According to the results of the paper chromatography, which amino acids were most attracted to the solvent? Which amino acids were most attracted to the paper? Explain.
2. According to your answer to question #1, which do you think is more polar, the chromatography solvent or the paper? Explain.
3. If you had completely hydrolyzed the egg albumin before doing the Biuret test on that sample, what results would you expect for the Biuret test? Explain why. Include what color you would expect to see, whether it is a positive or negative test, and what that means.
4. Suggest a reason why alcohol or other disinfectants are often applied to a person's skin before an injection is given (based on something you learned in this lab).
5. Draw the structure of phenylalanine in its regular form and its zwitterion form.
6. What substances react in the Biuret test?
7. What substances react in the Xanthoproteic test?
8. Suggest a reason why milk is used as an antidote for lead poisoning. Hint: milk contains a lot of protein.

9. Predict the results of each of the following reactions. Include the observed colors and whether it corresponds to a positive or a negative test.
   a. The Biuret test on proline
   b. The Biuret test on egg albumin
   c. The ninhydrin test on lysine
   d. The xanthoproteic test on egg albumin
   e. The sulfur test on cysteine