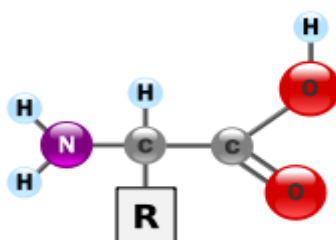


EXPERIMENT 2- QUALITATIVE ANALYSIS OF AMINO ACIDS AND PROTEINS

Amino acids are molecules containing **an amine group, a carboxylic acid group** and a side chain that varies between different amino acids. Amino acids of the general formula **$RCH(NH_2)COOH$** are amphoteric, behaving as amines in some reactions and as carboxylic acids in others. At a certain pH known as the **isoelectric point** an amino acid has no overall charge, since the number of protonated ammonium groups (positive charges) and deprotonated carboxylate groups (negative charges) are equal. Since the amino acids at their isoelectric points have both negative and positive charges, they are known as **zwitterions**.



Amino acids are critical to life. They have particularly important functions like being the **building blocks of proteins** and being the **intermediates in metabolism**.

Amino acids are generally classified by the properties of their side chain into four groups. The side chain can make an amino acid a weak acid or a weak base, and a hydrophile if the side chain is polar or a hydrophobe if it is nonpolar.

Proteins (also known as **polypeptides**) are organic compounds made of amino acids arranged in a linear chain. The amino acids in a polymer are joined together by the **peptide bonds** between **the carboxyl and the amino groups** of adjacent amino acid residues.

Like other biological macromolecules such as polysaccharides and nucleic acids, proteins are essential parts of organisms and participate in virtually every process within cells. **Proteins are important in:**

- catalyzing biochemical reactions (enzymes)
- structural and mechanical functions (actin and myosin)
- cell signaling
- immune responses
- cell adhesion
- cell cycle

TESTS ON AMINO ACIDS:

1) Solubility Tests:

The solubility of amino acids and proteins is largely dependent on the solution pH. The structural changes in an amino acid or protein that take place at different pH values alter the relative solubility of the molecule. In acidic solutions, both amino and carboxylic groups are protonated. In basic solutions, both groups are deprotonated.

Amino acids are essentially soluble in water. Their solubilities in water, dilute alkali and dilute acid vary from one compound to the other depending on the structure of their side chains. Apply this test to **glycine, tyrosine, glutamic acid and cysteine.**

Procedure:

- Note the solubility of amino acids in water and alcohol by placing a small amount in a test tube, adding a few mL of solvent and warming if necessary.
- Determine the amino acid solution is acidic or basic by using a litmus paper while testing the solubility in water.
- Repeat the solubility test using dilute HCl and dilute NaOH.

2) Ninhydrin Test:

Ninhydrin (triketohydrindene hydrate) is a chemical used to detect ammonia or primary and secondary amines. Amino acids also react with ninhydrin at pH=4. The reduction product obtained from ninhydrin then reacts with NH₃ and excess ninhydrin to yield a blue colored substance. This reaction provides an extremely sensitive test for amino acids. Apply this test to **any of the amino acids you choose.**

WARNING: Avoid spilling ninhydrin solutions on your skin, as the resulting stains are difficult to remove. (Ninhydrin is the most commonly used method to detect fingerprints, as the terminal amines or lysine residues in peptides and proteins sloughed off in fingerprints react with ninhydrin).

Procedure:

- To 1 mL amino acid solution add 5 drops of 0.2% ninhydrine solution in acetone.
- Boil over a water bath for 2 min.
- Allow to cool and observe the blue color formed.

Questions:

- ✓ Write the reaction(s) involved in Ninhydrin Test.

3) Stability to Alkali:

Amino acids, unlike amides and volatile amines, do not evolve NH_3 or alkaline vapor when boiled with alkali. This method can be used to differentiate amino acids from amines and amides. Apply this test to **the provided amine or amide and also to glycine.**

Procedure:

- Pipette 1 mL 1% glycine and the amide or amine solution into separate test tubes.
- Add 1 mL dilute NaOH to each test tube and boil.
- Test the vapor from each boiling tube with wet litmus paper.

Questions:

- ✓ What type of reaction is responsible for the evolution of alkaline vapor? Write the reaction and explain briefly.

4) Specific Reactions for Individual Amino Acids:

WARNING: Please DO NOT use vast amounts of solution for these tests, since most of the amino acids are very expensive!!

a) Xanthoproteic Test:

Some amino acids contain aromatic groups that are derivatives of benzene. These aromatic groups can undergo reactions that are characteristics of benzene and benzene derivatives. One such reaction is the nitration of a benzene ring with nitric acid. The amino acids that have **activated benzene ring** can readily undergo nitration. This nitration reaction, in the presence of activated benzene ring, forms **yellow** product. Apply this test to **tyrosine, tryptophan, phenylalanine and glutamic acid.**

Procedure:

- To 2 mL amino acid solution in a boiling test tube, add equal volume of concentrated HNO_3 .
- Heat over a flame for 2 min and observe the color.
- Now COOL THOROUGHLY under the tap and CAUTIOUSLY run in sufficient 40% NaOH to make the solution strongly alkaline.
- Observe the color of the nitro derivative of aromatic nucleus.

Questions:

- ✓ Write the reaction(s) involved in Xanthoproteic Test.
- ✓ Define "activated benzene ring", briefly.
- ✓ Do all the amino acids with aromatic side chains give positive result? Why?

b) Millon's Test:

Millon's test is specific to phenol containing structures (tyrosine is the only common phenolic amino acid). Millon's reagent is concentrated HNO_3 , in which mercury is dissolved. As a result of the reaction **a red precipitate or a red solution** is considered as positive test. A yellow precipitate of HgO is **NOT** a positive reaction but usually indicates that the solution is too alkaline. Apply this test to **tyrosine, phenylalanine, glycine and β -naphthol.**

Procedure:

- To 2 mL amino acid solution in a test tube, add 1-2 drops of Millon's reagent.
- Warm the tube in a boiling water bath for 10 min.
 - o A brick red color is a positive reaction.
 - o Note that this is a test for phenols, and the ninhydrin test should also be positive if it is to be concluded that the substance is a phenolic amino acid.

Questions:

- ✓ Write the reaction(s) involved in Millon's Test.
- ✓ You have phenol, tyrosine, cysteine and β -naphthol in separate test tubes. By using which test(s) would you find the tyrosine containing test tube? Explain, briefly.

c) Hopkin's Cole Test:

The indole group of tryptophan reacts with glyoxylic acid (glacial acetic acid, which has been exposed to light, always contains glyoxylic acid CHOCOOH as an impurity) in the presence of concentrated H_2SO_4 to give a purple color. Apply this test to **glycine, tryptophan and tyrosine.**

Procedure:

- To a few mL of glacial acetic acid containing glyoxylic acid, add 1-2 drops of the amino acid solution.
- Pour 1-2 mL H_2SO_4 down the side of the sloping test tube to form a layer underneath the acetic acid.
- The development of a purple color at the interface proves a positive reaction.

Questions:

- ✓ Write the reaction(s) involved in Hopkin's Cole Test.
- ✓ What is the role of H_2SO_4 in this test? Explain, briefly.

d) Lead-Sulfide Test:

When cystine is boiled with 40% NaOH, some of sulfur in its structure is converted to sodium sulfide (Na_2S). The Na_2S can be detected by using sodium plumbate solution which causes the precipitation of PbS from an alkaline solution. In order to apply this test, first the sodium plumbate solution should be prepared. Apply this test to **cysteine and cystine.**

Procedure:

- Sodium Plumbate Solution Preparation:
 - o Add 5 mL dilute NaOH to 2 mL dilute lead acetate.
 - o A white precipitate of lead hydroxide forms.
 - o Boil until the precipitate dissolves with the formation of sodium plumbate.
- Boil 2 mL amino acid solution with a few drops of 40% NaOH for 2 min.
- Cool and add a few drops of the sodium plumbate solution.
- A brown color or precipitate is a positive test for sulfides.

Questions:

- ✓ Write reaction(s) involved in the Lead-Sulfide Test.
- ✓ Explain what is “plumbate”?

e) Ehrlich Test:

Aromatic amines and many organic compounds (indole and urea) give a colored complex with this test. Apply this test to **tryptophan, urea and glycine.**

Procedure:

- Put 0.5 mL of the amino acid solution to a test tube.
- Add 2 mL Ehrlich reagent and observe the color changes.
- Repeat the test with urea solution.

Questions:

- ✓ What chemicals are found in Ehrlich's reagent.
- ✓ Explain the reaction involved in Ehrlich Test.
- ✓ Explain your observation for the urea solution when it is tested with Ehrlich's reagent.

f) Sakaguchi Test:

The Sakaguchi reagent is used to test for a certain amino acid and proteins. The amino acid that is detected in this test is arginine. Since arginine has a guanidine group in its side chain, it gives a red color with α -naphthol in the presence of an oxidizing agent like bromine solution. Apply this test to **arginine.**

Procedure:

- 1 mL NaOH and 3 mL arginine solution is mixed and 2 drops of α -naphthol is added.
- Mix thoroughly and add 4-5 drops of bromine solution **UNDER THE HOOD!!**
- Observe the color change.

Questions:

- ✓ Define and give the structure of guanidine.

g) Nitroprusside Test:

The nitroprusside test is specific for cysteine, the only amino acid containing sulfhydryl group (-SH). This group reacts with nitroprusside in the presence of excess ammonia. Apply this test **cysteine, cystine and methionin.**

Procedure:

- Put 2 mL amino acid solution into the test tube.
- Add 0.5 mL nitroprusside solution and shake thoroughly.
- Add 0.5 mL ammonium hydroxide.
- Observe the color change.

Questions:

- ✓ Write the reaction(s) involved in Nitroprusside Test.
- ✓ Is there any difference in the test results of cystine and cysteine? If there is, explain the reasons by giving the related structures.

5) Tests for Proteins:

a) Biuret Test:

The Biuret Test positively identifies the presence of proteins (not less than two peptides). The reaction in this test involves the complex formation of the proteins with Cu^{2+} ions in a strongly alkaline solution. Apply this test to **gelatin, casein and albumin.**

Procedure:

- To 2 mL protein solution, add 5-6 drops of dilute CuSO_4 (Fehling's solution A diluted 1/10 with water)
- Add 3 mL 40% NaOH solution.
- Observe the color change.

If the protein tested is insoluble in water, then apply the procedure given below:

- Measure 3 mL acetone and 1.5 mL water into a test tube.

- Add 1 drop of dilute NaOH and a little piece of protein to be tested.
- Boil continuously over a small flame for 2 min and cool.
- Add 0.5 mL 40% NaOH and 2 drops of a 1/10 diluted Fehling's solution A.
- Observe the color change.

Questions:

- ✓ Write the reaction(s) involved in Biuret's Test.

b) Ninhydrin Test:

This test is given by only amino acids and proteins which contain free $-NH_2$ groups in their structure. Apply this test for **all the proteins provided.**

c) Test for Amino Acids:

Perform the tests for individual amino acids on the **provided proteins.**

Xanthoproteic Test, Millon's Test, Hopkin's Cole Test, and Lead Sulphite Test.

Questions:

- ✓ According to your test results, indicate which amino acids are found on the protein structures that are tested.

d) Precipitation of Proteins:

The precipitation of a protein occurs in a stepwise process. The addition of a precipitating agent and steady mixing stabilizes the protein solution. Mixing causes the precipitant and the target product to collide. Enough mixing time is required for molecules to diffuse across the fluid.

I. By Neutral Salts:

The precipitation of a protein by neutral salt is commonly known as salting-out method. Addition of a neutral salt, such as ammonium sulfate, compresses the solvation layer and increases the protein-protein interaction. As the salt concentration of a solution is increased, more of the bulk water becomes associated with the ions. As a result, less water is available to take part in the solvation layer around the protein, which exposes hydrophobic parts on the protein surface. Therefore, proteins can aggregate and form precipitates from the solution. The amount of neutral salt required to cause protein precipitation varies with the nature of the protein and the pH of the solution. Apply this test to **all the proteins provided.**

Procedure:

- Add solid ammonium sulfate to about 5 mL of protein solution in a test tube (the salt should be added in quantities of approximately 1 g at a time)

- Agitate the solution gently after each addition to dissolve the ammonium sulfate.

Questions:

- ✓ The salting-out process occurs spontaneously. Can you explain the reason for this spontaneity with free energy, enthalpy and entropy concepts.

II. By salts of Heavy Metals:

Heavy metal salts usually contain Hg^{2+} , Pb^{2+} , Ag^{1+} , Tl^{1+} , Cd^{2+} and other metals with high atomic weights. Since salts are ionic, they disrupt salt bridges in proteins. The reaction of a heavy metal salt with a protein usually leads to an insoluble metal protein salt. Apply this test to **all the proteins provided.**

Procedure:

- Treat 3 mL of the protein solution provided with a few drops of mercuric nitrate.
- A white precipitate formation should be observed.

Questions:

- ✓ What would you expect to happen when you add mercuric nitrate on the solution of cystine amino acid? Explain, briefly.

III. By Acid Reagents:

The precipitation of a protein in the presence of acid reagents is probably due to the formation of insoluble salts between the acid anions and the positively charged protein particles. These precipitants are only effective in acid solutions. Apply this test to **all the proteins provided.**

Procedure:

- Treat 3 mL of protein solution provided with a few drops of trichloroacetic acid solution.
- Note the protein precipitate formed.

Questions:

- ✓ What could be the reason of using trichloroacetic acid as an acid reagent instead of commonly used ones?

6) Unknown Part:

- Take an unknown solid from your assistants and please **DO NOT forget to write your unknown number in your lab reports.**

- Carry out the amino acid and protein tests in a reasonable sequence to determine your unknown solid (**Please DO NOT trust on your solubility observations and physical appearances of your unknown**).