Chemical Test for Proteins and Amino Acids

Proteins

Proteins are class of organic compounds, which are present in and vital to every living cell. It is present in the form of skin, hair, callus, cartilage, muscles, tendons and ligaments; it protects and provides structure to the body of a multicellular organism. The **enzymes**, **hormones** and **antibodies** are made up of proteins, which catalyze and regulate the physiological activities of plant and human being. **Hemoglobins** and various lipoproteins transport oxygen and other substances within an organism. Protein deficiency may cause deficiency disease like kwasharkor. These are also toxic in nature like botulinum toxin, venom toxins, ricin and toxins produced by microorganisms like tetanus and diphtheria.

Classification of Proteins:

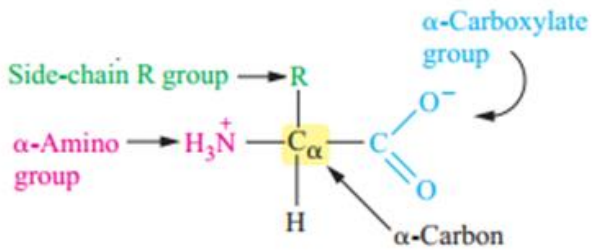
Proteins are classified mainly in two groups on the basis of their chemical nature and solubility.

1. **Simple proteins**: Composed of amino acids and soluble in water, dilute acid solutions, dilute alkali solutions, dilute salt solutions or in 70% alcohol. e.g. Albumins, globulins, glutelins, protamines and histones.
2. **Conjugate proteins**: Composed of other structural elements besides amino acids. e.g. chromoproteins, lipoproteins, metalloproteins and glycoproteins.
3. **Derived proteins:** In general plant proteins contain less content of Sulphur containing amino acids whereas plant cell wall is rich in hydroxyl proline.

Amino Acids

Amino acids are molecules containing an amine group, a carboxylic acid group and a side chain that varies between different amino acids. Amino acids are the building blocks of proteins. In humans, the amino acids used are α- amino acids,

which means the carboxylic acid group and the amino group are located on the same carbon. The general formula of Amino acids are **RCH (NH_2) COOH** and it's amphoteric, behaving as amines in some reactions and as carboxylic acids in others.

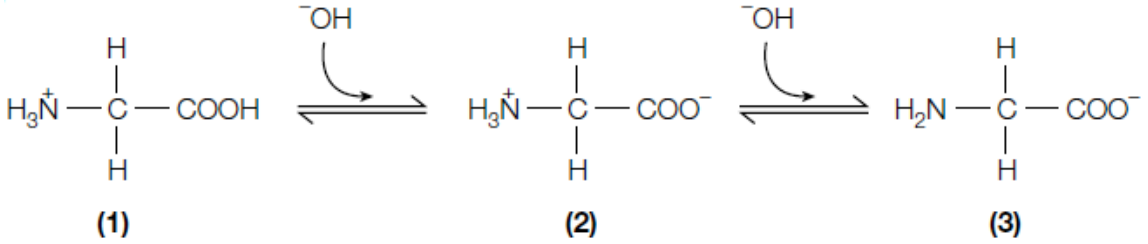


Hydrolysis of proteins by boiling aqueous acid or base yields α-amino acids. More than twenty such components have been isolated. Amino acids are classified generally in two categories on the basis of their synthesis in human body:

1. **Essential amino acids**: Essential component of diet since they are not synthesized by human body.
2. **Non-essential amino acids**: Synthesized in human body.

The α-amino and α-carboxyl groups on amino acids act as acid–base groups, donating or accepting a proton as the pH is altered. At low pH, both groups are fully protonated, but as the pH is increased first the carboxyl group and then the amino group loses a hydrogen ion.

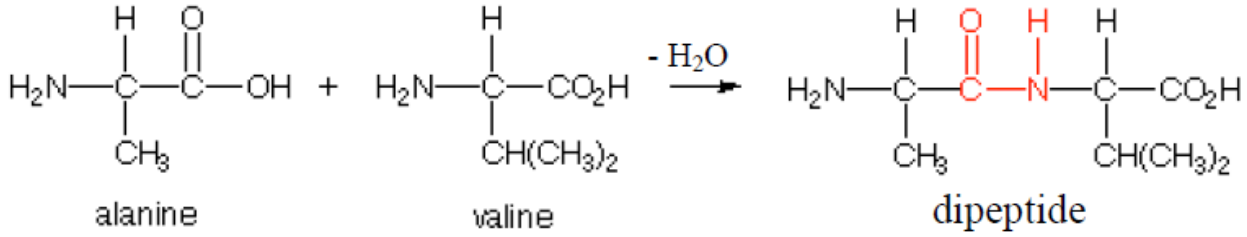
For the standard 20 amino acids, the pK is in the range 1.8–2.9 for the α-carboxyl group and 8.8–10.8 for the α-amino group. Those amino acids with an ionizable side-chain have an additional acid–base group with a distinctive pK.

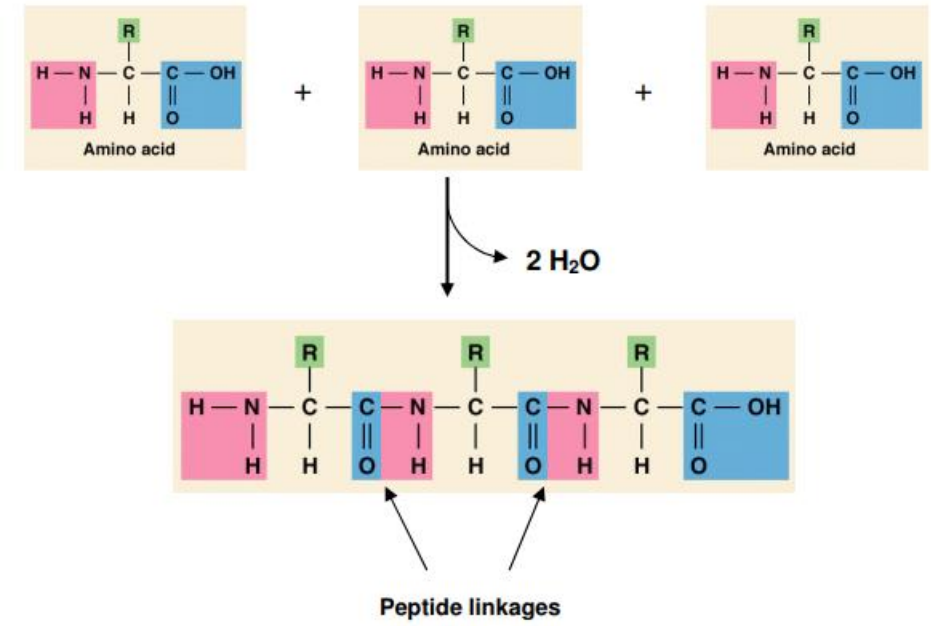


Isoelectric point (pI): The isoelectric point (pI) is the pH of an aqueous solution of an amino acid (or peptide) at which the molecules on average have no net charge. In other words, the negatively charged groups exactly balance the positively charged groups. The isoelectric point of an amino acid is the average of the pKa values of the protonation transitions on either side of the isoelectric species.

The pI simple amino acids like alanine, is the average of pKa's of carboxyl (2.34) and ammonium (9.69) groups. Hence, the pI of alanine can be calculated as: (2.34 + 9.69)/2 = 6.02. Or for aspartic acid isoelectric species exists in the pH domain between pKa1 (2.1) and pKa2 (3.9). Thus, the pI of aspartic acid is 2.1+3.9/2=3.0.

Reactive groups in amino acids include -NH_2 and -COOH groups and groups present on side R chains. In peptides and proteins only the side chain is available for reactions besides amino and carboxylic groups at the terminal ends. When amino acids are linked together, amide or peptide bonds are formed. The formation of an amide group is shown in the reaction below, in which two amino acids react to form a dipeptide.





Chemical Test for Proteins and Amino Acids

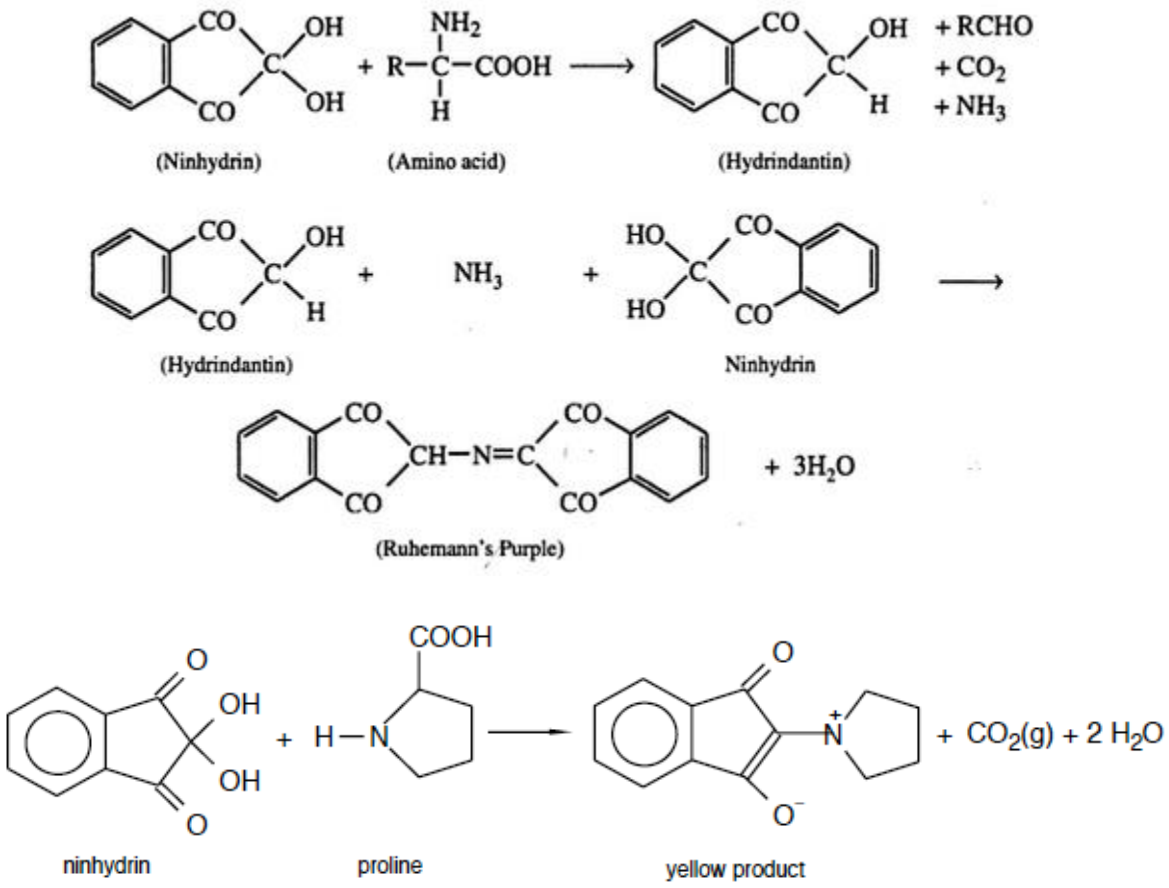
Proteins are high molecular weight polymers of amino acids. Amino acids are white compounds, more or less soluble in water and present in acid hydrolysates of plant and animal proteins.

A variety of color reactions specific to particular functional groups in amino acids are known. They are useful in both the qualitative and quantitative identification of particular amino acids.

Test one: Ninhydrin test (for all proteins and amino acids)

The Ninhydrin test is used to detect the presence of free α-amino acids and proteins containing free amino groups. Ninhydrin reacts with α-amino acids in two steps and two ninhydrin molecules react with one amino acid which gives characteristic deep blue or pale yellow color due to formation of complex between two ninhydrin molecule and nitrogen of free amino acid.. In the first step ninhydrin reacts with amino acids it itself gets reduced and the amino acid forms the corresponding

aldehyde and ammonia and carbon dioxide are released, in the next step the reduced ninhydrine and a fresh ninhydrin react with the ammonia and forms a colored complex is formed. Alpha amino acids react fast when compared to beta and gama. Imino amino acids give a different color.



Reagents:

1- Protein and amino acids.

2- Ninhydrin solution 2% (Ninhydrin dissolved in acetone).

Procedure:

1- Take 1 ml of protein or amino acid solution.

2- Add to it 10 drops of freshly prepared ninhydrin solution.

3- Boil the solution for 1 minute in a boiling water bath. Observation for the tubes Record the color.

All amino acids when heated with ninhydrin can form complexes: pink, purple, and blue- violet in color. The color complex is called ruhemanns purple. Proline and hydroxylproline, give yellow color.

Note

Ninhydrin is most commonly used as a forensic chemical to detect "fingerprints", as amines left over from proteins sloughed off in fingerprints react with ninhydrin giving a characteristic purple color

Warning

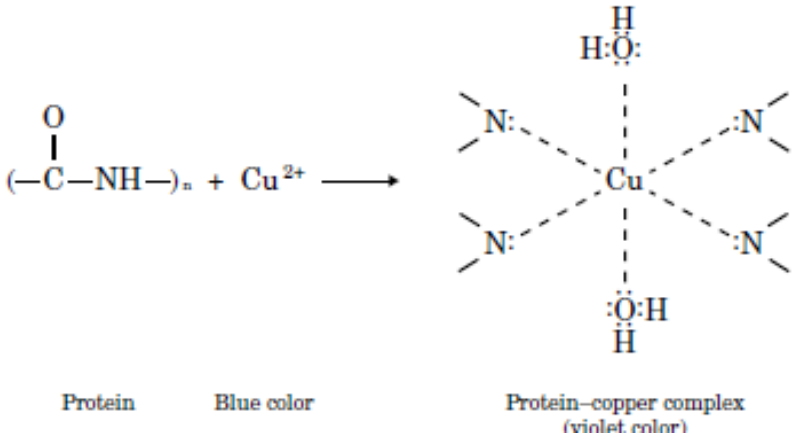
Ninhydrin is carcinogenic handle with care

Be careful not to spray ninhydrin on your hand and not to touch the sprayed areas with bare hands. If the ninhydrin spray touches your skin (which contains amino acids) your fingers will be discolored for a few days.

Test two: Biuret Test (for proteins and compounds containing two or more peptide bonds.

This is a general test for identification of proteins. This test is positive for all compounds containing two or more peptide bonds.

Protein (containing peptide bonds) reacts with copper (II) sulfate in Biuret solution ($CuSO_4$ and KOH) produce a copper complex, which has a violet color. The intensity of color is proportional to the number of peptide bonds. A deep violet color indicates the presence of proteins and a light pink color indicates the presence of peptides.



Color	Indication
Light blue	No protein or peptides
Violet	Protein
Pink	Peptides

Reagents:

1- Protein and amino acids.

2- NaOH solution 10%

3- $CuSO_4$ 1%

4- Acetic acid 20%.

Procedure:

1- Take 1 ml of protein or amino acid solution.

2- Add to it 1 ml of NaOH solution. Then add 1 drop of $CuSO_4$ solution while swirling. The development of a purplish-violet color is evidence of the presence of proteins.

3- Acidify the solution by Acetic acid.

At least two peptide linkages are necessary for this test; individual amino acids do not produce violet color.

Note

1- The Biuret method is a simple one-step process, and is the most widely used method for plasma protein estimations.

2- The sensitivity of the method is less and is unsuitable for estimation of proteins in milligram or microgram quantities.

The study of R- group of amino acids and proteins

A variety of color reactions specific to particular functional groups in amino acids are known. They are useful in both the qualitative and quantitative identification of particular amino acids.

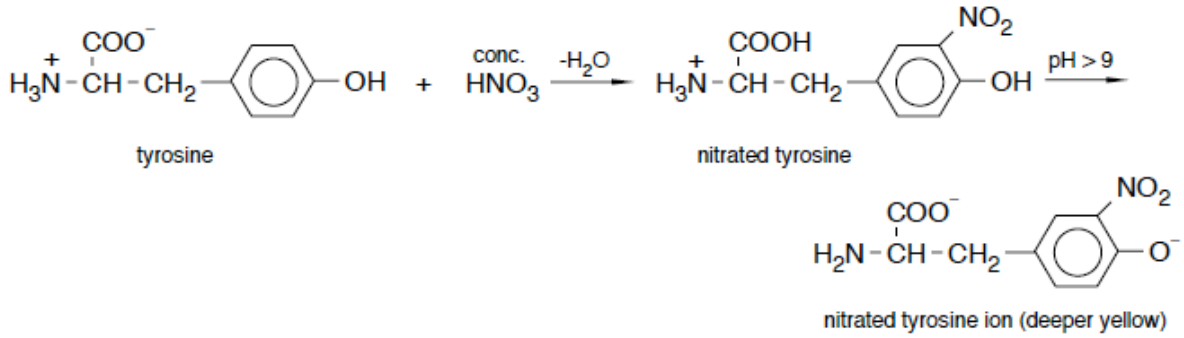
WARNING

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

Test three: Xanthoproteic test (Mulder's test) (for phenyl group)

This test gives positive result with aromatic amino acids containing phenyl groups in their structures like phenyl alanine, tyrosine and tryptophan.

This test is based on the ability of aromatic amino acids containing substituted phenyl groups to react with concentrated nitric acid to give yellow derivatives of nitrobenzene which turns to orange in alkaline medium.



Materials:

1. Test solution 1% (Amino acid or protein).
2. Concentrated nitric acid HNO_3.
3. Sodium hydroxide (10%).

Method:

1- Take 0.5 ml of test solution in a test tube.

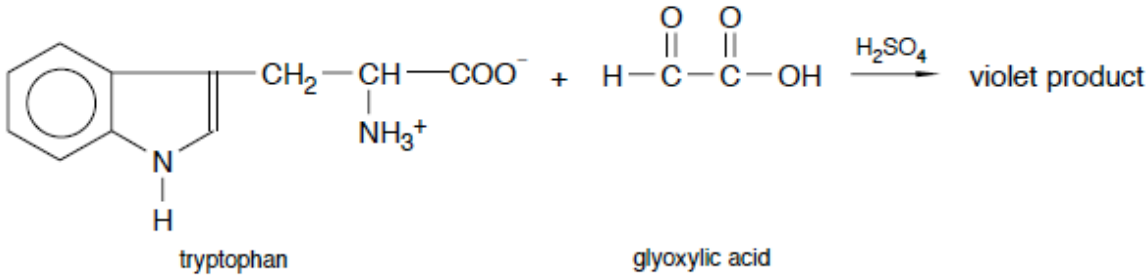
2- Add 0.5 ml of concentrated nitric acid. A white precipitate due to denaturation of protein is formed. Heat the test tubes carefully in a warm water bath for about three minutes. The appearance A yellow solution derivative is formed due to nitration of benzene ring.

3- Cool the test tube for 2-3 min by keeping under the tap and make it alkaline with 2 to 3 drops of sodium hydroxide drop by drop and mixing thoroughly till the yellow color is converted into an orange color. This confirms the presence of aromatic amino acids containing phenyl groups.

<u>Test four: Glyoxylic acid test (Hopkins-cole' reaction) (for indole group)</u>

This test is given by those amino acids, which have the indole groups. So, this test is specific for detecting tryptophan and is an indication of the presence of tryptophan in the protein.

The indole groups present in the tryptophan molecules react with glyoxylic acid in presence of con.H_2SO_4 forms a condensation product which is reddish violet, the sulfuric acid acts as dehydrating agent, eliminating a molecule of water. Tryptophan glyoxylic condensation product (reddish violet)



Reagents:

1. Test solution 0.5% (Amino acids like glycine, tyrosine, tryptophan).
2. Glacial acetic acid which has been exposed to the light for a few days.
3. Concentrated H_2SO_4

Procedure:

1- Take 1ml of the test solution in a test tube

2- Add 1 ml of glyoxylic (Glacial acetic acid which has been exposed to the light) mix the solution.

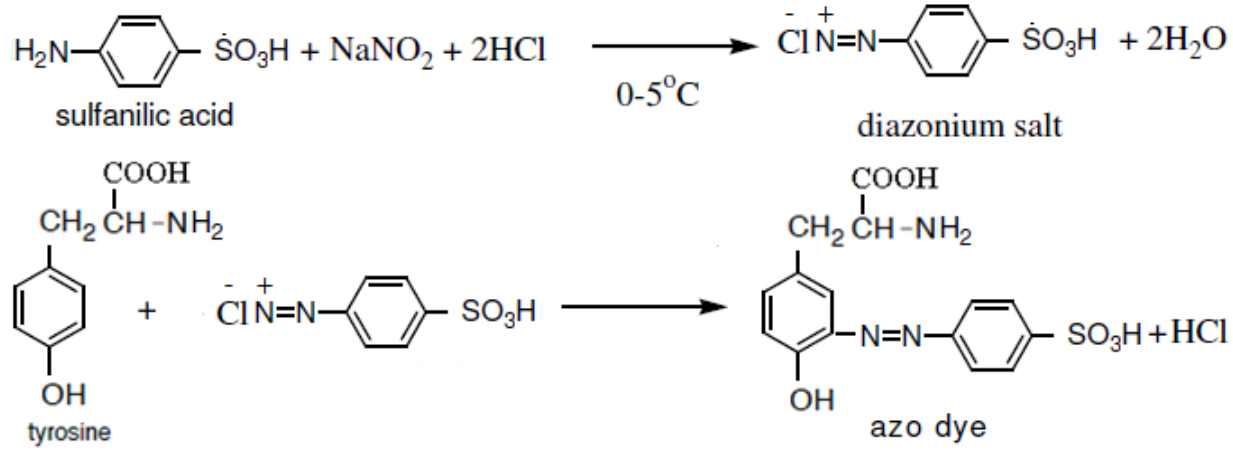
3- Then carefully pour around 1 ml of concentrated H_2SO_4 to the side of the test tube (do not shake the test tube, while adding acid). The two distinct layers are formed. The appearance of a violet ring at the junction of the two layers indicates the presences of tryptophan.

Note

Care should be taken while using H_2SO_4

Test five: Pauly's test (For imidazole group and phenol groups in amino acids)

This test is specific for the detection of imidazole group in tryptophan and phenol group in tyrosine. The reagent used for this test contains sulphanilic acid dissolved in hydrochloric acid. Sulphanilic acid upon diazotization in the presence of sodium nitrite and hydrochloric acid results in the formation a diazonium salt. The diazonium salt formed, couples with either tyrosine or histidine in alkaline medium to give a red colored (azo dye).



Reagents:

1. Test solution 0.5% (Amino acids and protein).
2. Sulphanilic acid 1%
3. Concentrated HCl 2%
4- Sodium carbonate Na_2CO_3 (1%)
5- Sodium nitrite $NaNO_2$ (0.5%).

Procedure:

1- Take 1ml of (1% sulphanilic acid dissolved in 2% HCl) in test tube.

2- Add 1 ml of sodium nitrite, mix and cool the solution. Wait for 1 minute.

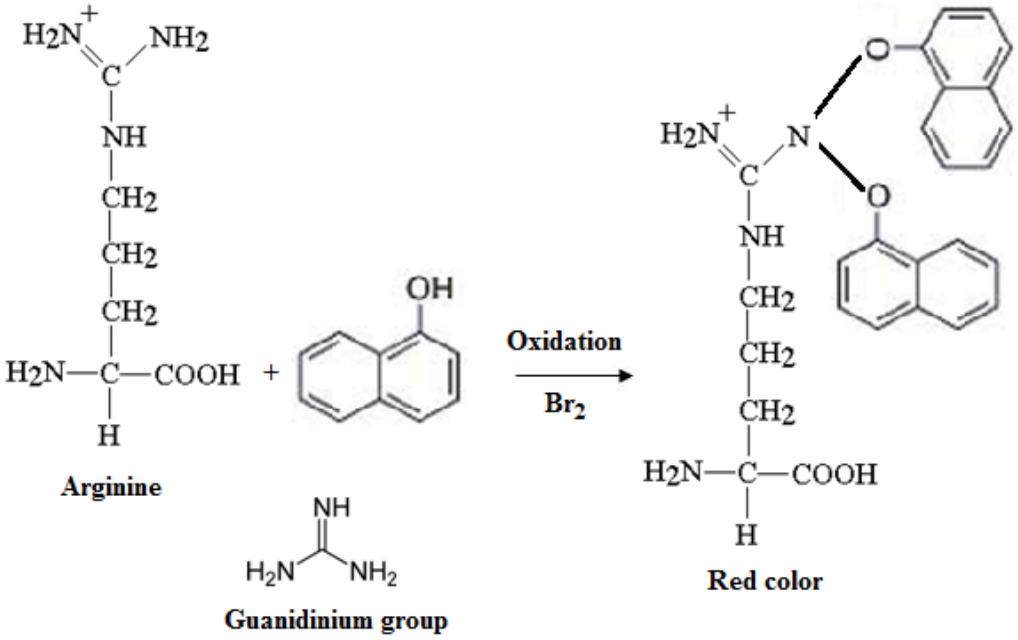
3- Then pour 1 ml of test solution. Mix well, and then add 2 ml of sodium carbonate until the color appears. The formation of cherry red color or orange red color indicates the presence of histidine and phenol group in tyrosine.

Note

Diazobenzene sulphonic acid reacts with the imidazole ring of histidine or the hydroxyl group in phenol.

Test six: Sakaguchi test (for guanidino group)

This test is given by all compounds containing guanidine group and thus is an indication of the presence of free arginine or arginyl residues in proteins. Free Arginine or arginyl residues in proteins react with α-naphthol in alkaline hypobromide solution to give bright red color. This is due to the presence guanidine group.



Reagents:

1- Test solution 0.5% (Amino acids like glycine, tyrosine, tryptophan).

2- Sodium hydroxide (5%).

3- α-Naphthol 1% (α-naphthol dissolved in ethanol).

4- Bromine water (10%).

Procedure:

1- Put 1ml of the test solution in a test tube.

2- Add 1 ml of sodium hydroxide, and 2 drops of ethanolic α-naphthol and 2 drops of bromine water reagent. Mix well. Wait for 5 minutes. Development of bright red color takes place this indicates the presence of arginine of proteins containing arginine.

Note
Other substances containing a guanidine group, such as creatine, also respond to this test.

Test seven: Lead acetate Test (test for S-S and SH)

Sulphur is present in cystine, cysteine, and methionine. This test is specific for sulphur containing amino acids cystine and cysteine but not methionine. On heating, cysteine and cystine react with sodium hydroxide (hot alkali) yield sodium sulphide. This reaction is due to partial conversion of the organic sulphur to inorganic sulphide. Then sodium sulphide reacts with lead acetate to give black precipitate of lead sulfide.

Reagents:

1- Test solution 0.5% (Amino acids or protein).

2- Sodium hydroxide (40%).

3- Lead acetate solution 10%

Procedure:

1. Put 2 ml of the test solution in a test tube.

2. Add 2 ml of sodium hydroxide (to make the solution strongly alkaline) and boil the contents for 5min over a Bunsen burner.

3. Cool the contents and add 10 drops of lead acetate solution and observe the color. A black or brown precipitate of PbS insoluble in HCl is formed indicated the presence of cysteine or cystine.

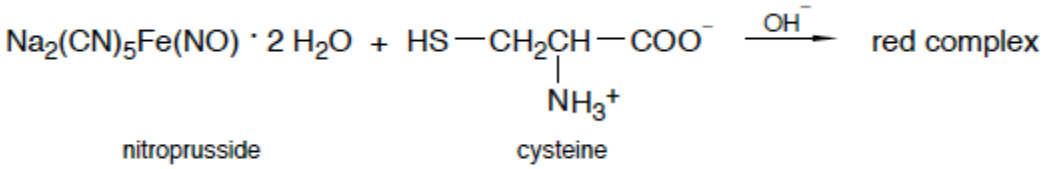
Note

1- Avoid the excess of lead acetate solution which will form white precipitate.

2- Methionine does not answer this test because sulphur in methionine is in the thio- ether linkage which is difficult to break by treatment with NaOH. Albumin (found in blood or egg) and keratin (found in skin, nail, and hair) will answer sulphur test positively because contain high proportional of cysteine and cystine; but casein (found in milk) will give a negative test because contain low proportional of cysteine and cystine.

Test eight: Nitroprusside test (for SH group)

This is a specific test for amino acids containing free thiol group like cysteine. Thiol group of cysteine can react with sodium nitroprusside [$Na_2Fe(CN)_5NO$] to give in the presence of excess amount of ammonia to give a violet color.



Reagents:

1- Test solution 0.5% (Amino acids or protein).

2- Sodium nitroprusside (2%).

3- Ammonium sulphite.

4- Ammonia solution.

Procedure:

1- Put 2 ml of the ammonium sulphate solution in a test tube.

2- Add 2 ml of test solution, followed by 0.5 ml of freshly prepared solution of sodium nitroprusside.

3- Pour 0.5 ml of liquor ammonia solution. Appearance of violet color indicates the presence of cysteine.

Questions

How you can distinguish between proteins and amino acids with other compounds

How you can distinguish between albumin and fructose

How you can distinguish between amino acids and glucose

How you can distinguish between Cysteine and cystine

Methionine give negative result with lead acetate test, why?

How you can distinguish between arginine and glycine (or other amino acids)

How you can distinguish between tryptophan and other amino acids

How you can distinguish between tyrosine and other amino acids

How you can distinguish between (phenyl alanine, tyrosine and tryptophan) and other amino acids

Write the reaction of Ninhydrin with any amino acids.

Write the reaction(s) involved in Xanthoproteic Test.

Do all the amino acids with aromatic side chains give positive result with Xanthoproteic Test? Why?

Write the reaction(s) involved in Glyoxylic acid test Test.

What is the role of H_2SO_4 in Glyoxylic acid test? Explain, briefly.

Write reaction(s) involved in the Lead-acetate Test.

Define and give the structure of guanidine.

Write the reaction(s) involved in Nitroprusside Test.

Is there any difference in the test results of cystine and cysteine in Nitroprusside Test? If there is, explain the reasons by giving the related structures.

Write the reaction(s) involved in Biuret's Test