QUALITATIVE TEST FOR PROTEINS

Introduction

As the name suggest amino acids are organic compounds that contain amino and carboxyl groups. The R- in the above formula stands for different chemical groups (may be aliphatic, aromatic or heterocyclic) and this determines the characteristics of the amino acids.The color tests have frequently been used for qualitative detection of amino acids. Not all amino acids contain the same reactive groups. For this reason the various color tests yield reactions varying in intensity and type of color according to the nature of groups contained in the particular amino acid under examination.

1. Millon's reaction

Principle

The reaction is due to the presence of the hydroxyphenyl group, C_6H_5OH in the amino acid molecule; and any phenolic compound which is unsubstituted in the 3,5 positions such as tyrosine, phenol and thymol will give the reaction. Solutions of nitric acid containing mercuric nitrate reacts with phenols, producing red colors or yellow precipitates which react with nitric acid to form red solution. The reaction probably depends on the formation of a nitro compound; which then reacts with phenol.

Materials

1. Millon's reagent

19.2.1.3 Method

Add 3 to 4 drops of Millon's reagent to 5 ml of test solution. Mix and bring the mixture gradually to a boiling point by heating over a small flame. Development of red color is due to the presence of protein. Excess of reagent should however be avoided since it may produce a yellow color which is not a positive reaction.

2. Xanthoproteic reaction

Principle

This reaction is due to the presence in the amino acid molecule of the phenyl group $–C_6H_5$, with which the nitric acid forms certain nitro modifications. The particular amino acids which are of especial importance in this connection are those of tyrosine and tryptophan. Phenylalanine does not respond to this test as it is ordinarily preformed.

Materials

1. Conc. HNO_3
2. Ammonium hydroxide
3. Sodium hydroxide

Method

Add 1 ml of conc. Nitric acid to 2 to 3 ml of test solution in a test tube. A white precipitate forms, which upon heating turns yellow and finally dissolves, imparting to the solution a yellow color, cool the solution and carefully add ammonium hydroxide or sodium hydroxide in excess. Note that the yellow color deepens into an orange.

3. Hopkins-Cole reaction

Principle

The formation in this test color is due to the presence of indoyl group. Gelatin does not respond to this test due to lack of amino acid tryptophan. Violet to blue colors develop when a mixture of protein and an aldehyde is layered over conc. sulphuric acid. A number of tests based on this principle have been suggested; all depends on the presence of the indoly group of tryptophan which reacts as follows (using glyoxylic acid as an example of an aldehyde).

This is called Hopkin-Cole test- A similar test was at one time recommended for detection of formaldehyde that had been as a preservative to milk, the formaldehyde reacting with indolyl groups of milk proteins to give a color.

Materials

1. Hopkin-Cole reagent
2. Conc. H_2SO_4

Method

Place 2 to 3 ml of test solution and an equal volume of Hopkins-Cole reagent in a test tube and mix thoroughly. Incline the tube and permit 5 to 6 ml of conc. sulphuric acid to flow slowly down the side of the tube, thus forming a sharp layer of acid beneath the amino acid solution. When stratified in this manner a reddish-violet color forms at the zone of contact of the two fluids. If the color does not appear after starting for a few minutes, the tube may be rocked gently to cause a slight mixing of the liquids are mixed by gentle stirring the precipitate of protein dissolves and the violet color spread throughout the solution.

4. Biuret test

Principle

The Biuret test is given by those substances whose molecules contain two cabamyl ($-CONH_2$) groups joined either directly or through a single atom of nitrogen or carbon. Similar substances which contain- $CSNH_2$,

-C(NH)NH_2, or – CH_2NH_2 in place of the –$CONH_2$ group also respond to the test. It follows from this fact that substance which are non-protein in character but which contain the necessary groups will respond to the biuret test.

Protein responds positively since there are pairs of CONH groups in the molecule. A copper coordination complex with the ring structure is probably produced. Short chain polypeptides give a pinkish violet color, longer one including proteins a more purple blue. The amino acid histidine gives a pink color, which depends on the peptide linkage, does not vary greatly in intensity from protein to protein. Several procedure based on this method have been suggested for quantitative determination of milk proteins, but it is not in general use in dairy research.

Materials

1. 10% NaOH
2. 0.5% $CuSO_4$

Method

To 2 to 3 ml of test solution in a test tube add an equal volume of 10% sodium hydroxide solution, mix thoroughly, and add a 0.5% copper sulphate solution drop by drop, mixing between drops, until a purplish-violet or pinkish-violet color is produced. The color depends upon the nature of the protein, proteoses and peptones give a decided pink; the color produced with gelatin is not far removed from a blue.

5. Ninhydrin reaction

Principle

This test gives positive results with proteins, peptones, peptides, amino acids and other primary amines, including ammonia. Proline and hydroxyproline give yellow color with ninhydrin, while other acids give blue to purple color.

Materials

1. 0.1% Ninhydrin
2. pH paper

Method

To 5 ml of dilute test solution, which must be approximately between pH 5 and pH 7 (a few drops of pyridine or a few crystals of sodium acetate may be used to adjust the pH), add 0.5 ml of 0.1 % ninhydrin, heat to boiling for one to two minutes, and allow to cool. A blue color develops if the test is positive.

6. Folin test

A phosphomolybdotungstic acid reagent designed by Folin for phenol has been widely used for detection and analysis of indolyl and phenol groups in amino acids. A characteristic blue color is formed when amino acid solution is warmed with this reagent. The color so formed is due to the reaction of alkaline copper with the amino acid and the reduction of phosphomolybdate by tyrosine and tryptophan present.

Materials

1. Alkaline Na_2CO_3 solution (2% in 0.1 N NaOH)
2. $CuSO_4$-Na; K tartarate solution (0.5 % $CuSO_4$) in 1 % Na, K tartarate) prepared fresh by mixing stock solutions.
3. “Alkaline solution” (prepared by mixing 50 ml of the reagent (1) and 1 ml of the reagent (2)).
4. Folin-Ciocalteau reagent

Method

Add 5ml of the alkaline solution to 1 ml of the test solution. Mix thoroughly and allow to stand at room temperature for 10 mins. Add 0.5 ml diluted Folin-Ciocalteau reagent rapidly with immediate mixing. Observe for development of color after 30 mins. Development of characteristic blue color indicates presence of indolyl or phenol group.

7. Sakaguchi test

Principle

Arginine and other guanidyl derivatives (glycocyamine, methylgyanidine etc) react with hypo bromide and alpha napthol to give a red colored product.

Materials

1. Sodium hydroxide solution (40%)
2. Alpha napthol solution (1% in alcohol)
3. Bromine water (a few drops of bromine in 100 ml distilled water)

Method

Mix 1 ml of sodium hydroxide with 3 ml of test solution and add 2 drops of alpha napthol. Mix thoroughly and add 4 to 5 drops of bromine water. Note the color formed. Formation of a red color indicates presence of guanidine group. This is a very sensitive and specific test.

8. Nitroprusside test

Principle

Sodium nitroprusside reacts with compounds containing sulphahydryl groups produce an intensely red but somewhat unstable color.

Materials

1. Sulphur amino acids (1.0% cystine, cysteine and methionine)
2. Sodium nitroprusside (2% prepared fresh)
3. Ammonium hydroxide

Method

Mix 0.5 ml of a fresh solution of sodium nitropruside with 2 ml of the test solution and add 0.5 ml of ammonium hydroxide.

Spectrophometric method

The use of infrared and ultraviolet spectra offers a mean of identification of amino acids and their derivatives. Spectrophometric method of detecting and determining amino acids in the intact protein offer advantages over chemical methods in that they do not involve hydrolysis; which often leads to partial decomposition of some amino acids.

The absorption of UV radiation at wave lengths of 280 nm, can be used as a method for detecting and determining some amino acids content. The light is absorbed by the amino acids tyrosine, tryptophan and phenylalanine.**Estimation of**

9. Protein by Lowry's method (Quantitative)

Objective : To estimate the protein using Lowry's method.

Principle: The –CO-NH- bond (peptide) in polypeptide chain reacts with copper sulphate in an

alkaline medium to give a blue colored complex. In addition, tyrosine and tryptophan residues of

protein cause reduction of the phosphomolybdate and phosphotungstate components of the

Folin-Ciocalteau reagent to give bluish products which contribute towards enhancing the

sensitivity of this method.

Reagents Required:

1. Reagent A: 2% sodium carbonate in 0.1 N sodium hydroxide.

2. Reagent B: 0.5% copper sulphate (CuSO4.5H2O) in 1% potassium sodium tartarate. Prepare

fresh by mixing stock solutions.

3. Alkaline copper solution (Reagent C): Mix 50mL of reagent A and 1 mL of reagent B prior

to use.

4. Diluted Folin’s reagent (Reagent D): Dilute Folin-Ciocalteau reagent with an equal volume

of 0.1 N NaOH

5. Standard: Dissolve 50mg BSA in 50mL of distilled water in a volumetric flask. Take

10mL of this stock standard and dilute to 50 mL in another flask for working standard

solution. One mL of this solution contains 200 µg protein.

Apparatus and Glass wares required: Test tubes, Pipettes, Colorimeter, etc.,

Procedure:

1. Pipette out 0.2, 0.4, 0.6, 0.8 and 1 ml of working standard in to the series of labeled test

tubes.

2. Pipette out 1 mL of the sample in another test tube.

3. Make up the volume to 1 mL in all the test tubes. A tube with 1 mL of distilled water

serves as the blank.

4. Now add 5 mL of reagent C to all the test tubes including the test tubes labeled 'blank'

and 'unknown'.

5. Mix the contents of the tubes by vortexing / shaking the tubes and allow to stand for 10

min.

6. Then add 0.5 mL of reagent D rapidly with immediate mixing well and incubate at room

temperature in the dark for 30 min.

7. Now record the absorbance at 660 nm against blank.

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8. Then plot the standard curve by taking concentration of protein along X-axis and

absorbance at 660 nm along Y-axis.

9. Then from this standard curve calculate the concentration of protein in the given sample.

Objective:

To characterize carbohydrates present in an unknown solution on the basis of various chemical assays

1- Molisch Test: specific for carbohydrates.

2- Benedict's Test: presence of reducing sugars.

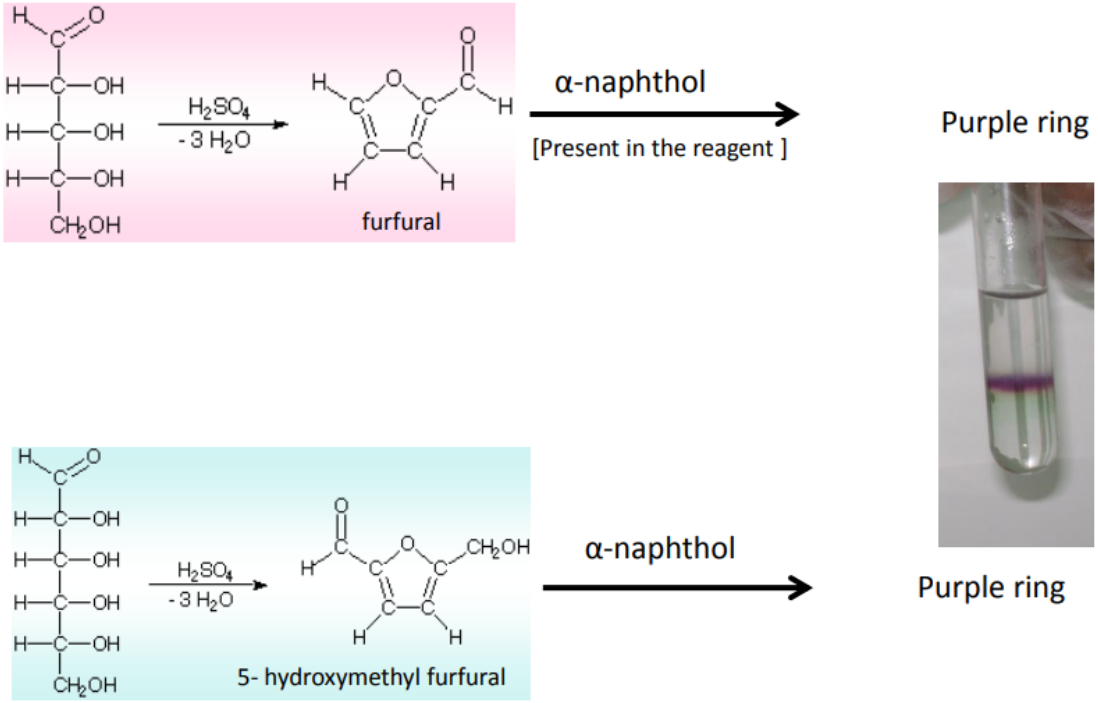
3- Barfoed's Test: test used for detecting the presence of monosaccharides.

4- Bial's Test: used to detect pentose [5C] monosacharides.

5- Seliwanoff's Test: distinguish between aldoses and ketoses.

1. Molisch's Test:

This is a common test for all carbohydrates larger than tetroses. The test is on the basis that pentoses and hexoses are dehydrated by conc. Sulphuric acid to form furfural or hydroxymethylfurfural, respectively. These products condense with α-naphthol to form purple condensation product.

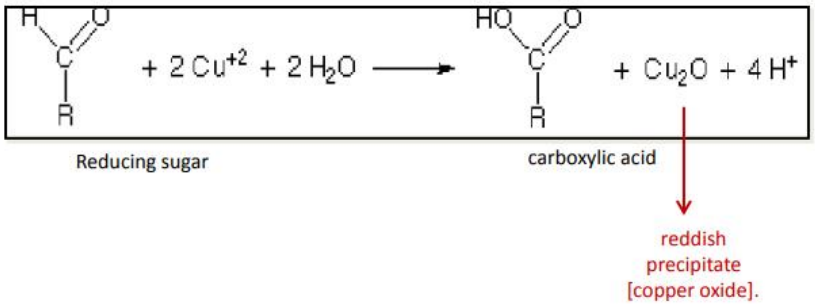


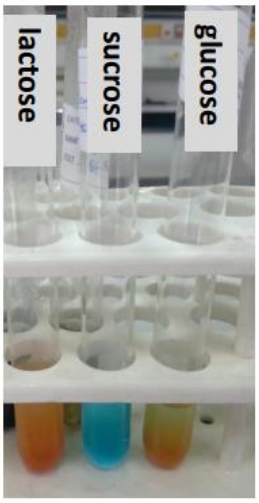
2. Fehling's Test:

This forms the reduction test of carbohydrates. Fehling's solution contains blue alkaline cupric hydroxide solution, heated with reducing sugars gets reduced to yellow or red cuprous oxide and is precipitated. Hence, formation of the yellow or brownish-red colored precipitate helps in the detection of reducing sugars in the test solution.

3. Benedict's Test:

As in Fehling's test, free aldehyde or keto group in the reducing sugars reduce cupric hydroxide in alkaline medium to red colored cuprous oxide. Depending on the concentration of sugars, yellow to green color is developed . All monosaccharides are reducing sugars as they all have a free reactive carbonyl group. Some disaccharides, like maltose, have exposed carbonyl groups and are also reducing sugars, but less reactive than monosaccharides





4. Barfoed's Test:

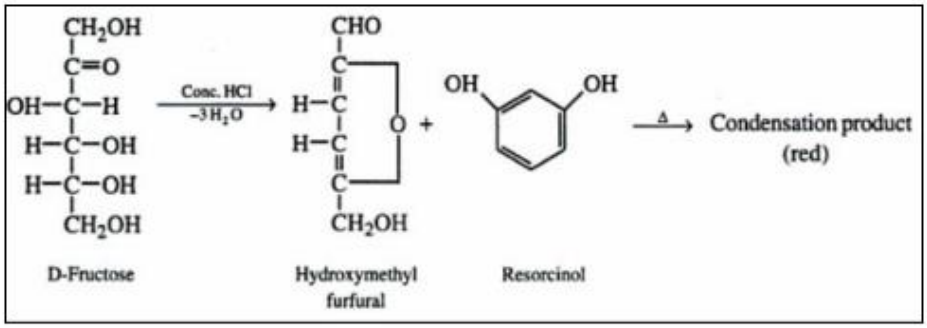
Barfoed's test is used to detect the presence of monosaccharide (reducing) sugars in solution. Barfoed's reagent, a mixture of ethanoic (acetic) acid and copper(II) acetate, is combined with the test solution and boiled. A red copper(II) oxide precipitate is formed will indicates the presence of reducing sugar. The reaction will be negative in the presence of disaccharide sugars because they are weaker reducing agents. This test is specific for monosaccharides . Due to the weakly acidic nature of Barfoed's reagent, it is reduced only by monosaccharides.

5. Seliwanoff's Test:

It is a color reaction specific for ketoses. When conce: HCl is added. ketoses undergo dehydration to yield furfural derivatives more rapidly than aldoses. These derivatives form complexes with resorcinol to yield deep red color. The test reagent causes the dehydration of ketohexoses to form 5-hydroxymethylfurfural. 5-hydroxymethylfurfural

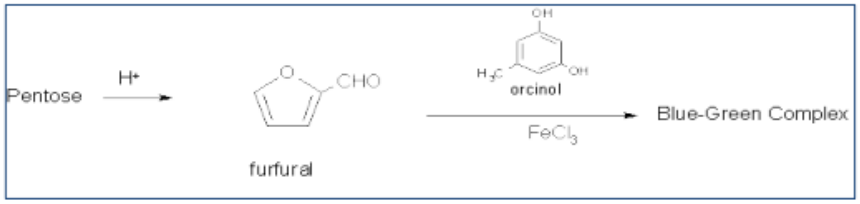
reacts with resorcinol present in the test reagent to produce a red product within two minutes . Aldohexoses reacts so more slowly to form the same product.

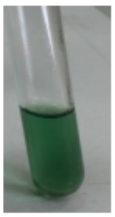
Seliwanoff's Test:

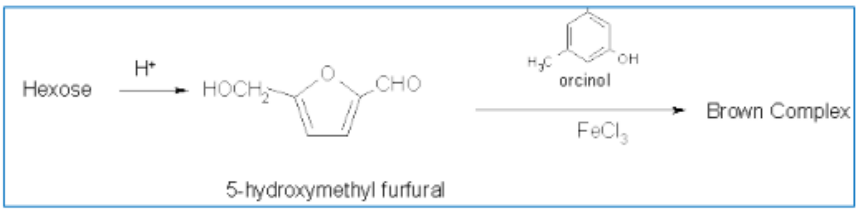


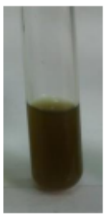
6. Bial's Test:

Bial's test is used to distinguish between pentoses and hexoses. They react with Bial's reagent and are converted to furfural. Orcinol and furfural condense in the presence of ferric ion to form a colored product. Appearance of green colour or precipitate indicates the presence of pentoses and formation of muddy brown precipitate shows the presence of hexoses.









7. Iodine Test:

This test is used for the detection of starch in the solution. The blue-black colour is due to the formation of starch-iodine complex. Starch contain polymer of α-amylose and amylopectin which forms a complex with iodine to give the blue black colour.

8. Osazone Test:

The ketoses and aldoses react with phenylhydrazine to produce a phenylhydrazone which further reacts with another two molecules of phenylhydrazine to yield osazone. Needle-shaped yellow osazone crystals are produced by glucose, fructose and

mannose, whereas lactosazone produces mushroom shaped crystals. Crystals of different shapes will be shown by different osazones. Flower-shaped crystals are produced by maltose.

- Why sucrose gives negative Benedict test?
- Explain, although starch has free hemiacetal bond it gives negative Benedict test?
- Why glucose (monosaccharide) and maltose (disaccharide) give positive Benedict
- test?
- What is the difference between Benedict and Barfoed's reaction?
- What are the carbohydrates' that give positive result with Seliwanoff ? why?

ESTIMATION OF DNA BY DIPHENYLAMINE REACTION

Principle:

The deoxyribose in DNA in the presence of acid forms β-hydroxylevulinaldehyde which reacts with diphenylamine to give a blue colour with a sharp absorption maximum at 595nm. In DNA, only the deoxyribose of the purine nucleotides react, so that the value obtained represents half of the total deoxyribose present.

METHODOLOGY

a) Materials required:

1. Equipments:

• Spectrophotometer

• Water bath

2. Chemicals/reagents:

• Standard DNA solution (0.25mg/ml)

• Diphenylamine reagent

• DNA sample in saline citrate buffer

• Saline citrate buffer (0.15M NaCl, 0.015M sodium citrate, pH 7.0)

• Glacial acetic Acid

• Concentrated H_2SO_4

• Ethanal

3. Glasswares and others:

• Test tubes

• Pipettes

• Graduated cylinder

b) Procedure:

Preparation of reagent: Dissolve 1.5g diphenylamine in 100ml of glacial acetic acid. Add 1.5ml of conc H_2SO_4. Store the solution in a dark glass bottle. On the day of use, prepare a fresh solution of ethanal (1ml) in dH_2O (50ml). Add 0.5ml of this solution to

each 100ml of the diphenylamine solution.

Caution: Wear eye protection and use a fume cupboard when preparing this reagent. Diphenylamine is harmful if ingested or inhaled and may irritate skin or eyes if it comes into contact with them.

c) Assay:

1. Prepare a series of dilutions of standard DNA (0.25mg/ml) in saline citrate buffer to give a concentration of 50-500µg/ml.

2. Prepare all the samples in triplicate.

3. To 2ml of each dilution of blank, standard and unknown add 4ml of diphenylamine reagent and mix. Tube1 is used as blank and tubes 2 through 7 are used for construction of a standard calibration curve for DNA. Tubes 8-11 are for unknown samples. (Table1)

4. Incubate all the tubes in boiling water for 10 min.

5. Cool the tubes and read the absorbance at 595nm against the blank.

6. Construct a standard curve of absorbance A595 vs. quantity of DNA and then calculate the concentration of unknown DNA dissolved in the saline citrate solution.

Calculation: Determine the amount of DNA in the unknown sample by plotting a standard curve of A595 on Y-axis and µg of DNA on X-axis.

References:

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2. J. Jayaram. (1981) Laboratory Manual in Biochemistry. New Age International Ltd. New Delhi.

3. Burton, K. (1956) A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochemical Journal. 62: 314-323.