Qualitative tests of amino acids and proteins and enzyme kinetics

Prepared by

Author

Alyaa H. Al-Mossawi

Dr. Zahraa S. Al-Garawi

Mustansiriyah University
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Qualitative Tests of Amino Acids and Proteins, and Enzyme Kinetics

إعداد
علياء هاشم الموسوي
م. كيمياء حياتية

إعداد وتاليف
د. زهراء سالم الكرعاوي
أ. م. كيمياء حياتية
z.mosin@uomustansiriyah.edu.iq

الجامعة المستنصرية

تم طبع هذا المؤلف في مكتبة حسنين للطباعة والاستنساخ، شارع فلسطين مقابل الجامعة المستنصرية.
رقم الهاتف 07707643338
البريد الالكتروني doublem985@gmail.com

الطبعة الاولى في شباط ٢٠١٨
حُدّث في ايلول ٢٠١٨
بغداد

شكـــــــر وتقديـــــــر

نتقدم بالشكر والامتنان الى الأساتذة الأعزاء في قسم الكيمياء، الجامعة المستنصرية أ.م.د. فلاح سموم الفرطوسي (رئيس فرع الكيمياء حياتية) و أ.م.د. حسنين كامل البيرماني، للتأييد والمساندة وتقديم المقترحات القيّمة لإنجاز هذا المؤلف المنهجي.

Contents

Preface

This handbook is for the 3rd year students of the Chemistry Department at Mustansiriyah University. It has the syllabus for the second course of the Biochemistry lab. The handbook contains some qualitative tests for detecting the amino acids and proteins, as well as, the enzyme kinetic experiments, which are currently available in our lab. The qualitative tests are commonly used to detect different types of amino acids and proteins either separately or within a mixture.

The last part of this handbook contains the enzyme kinetics experiments, which are used to determine the kinetic parameters of the

enzyme alkaline phosphatase in the presence and absence of inhibitor.

The test methods in this handbook have been qualified as standard methods using valid reagents. Some methods here employ toxic or very acidic solutions which need to be handled with care. Safety protectors such as eye wears, gloves, lab coats are important for students and lab staff to do each experiment. Using this handbook by the students is necessary to follow the experiments, to complete the homework and to study for the exams.

Dr. Zahraa
https://uomustansiriyah.edu.iq/e-learn/profile.php?id=4801

Qualitative tests of amino acids

Amino acids

An amino acid (R-CH-NH_2COOH) contains an amino group and a carboxylic acid group as side chains. It is the building block of all proteins and is linked with other amino acids as a chain by the peptide bonds (CONH-) to form the primary structure of a protein, see Figure 1.

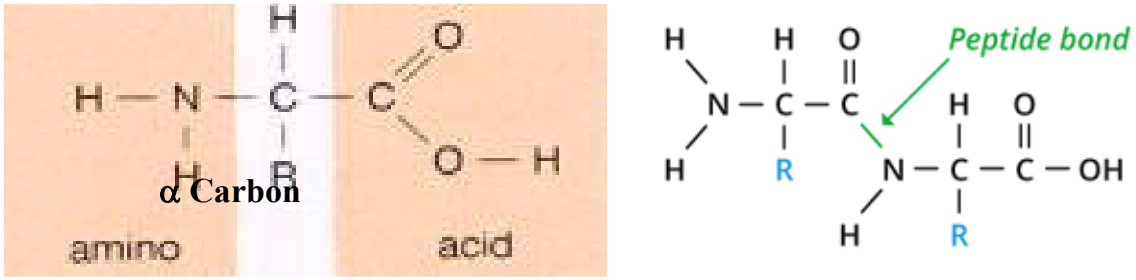
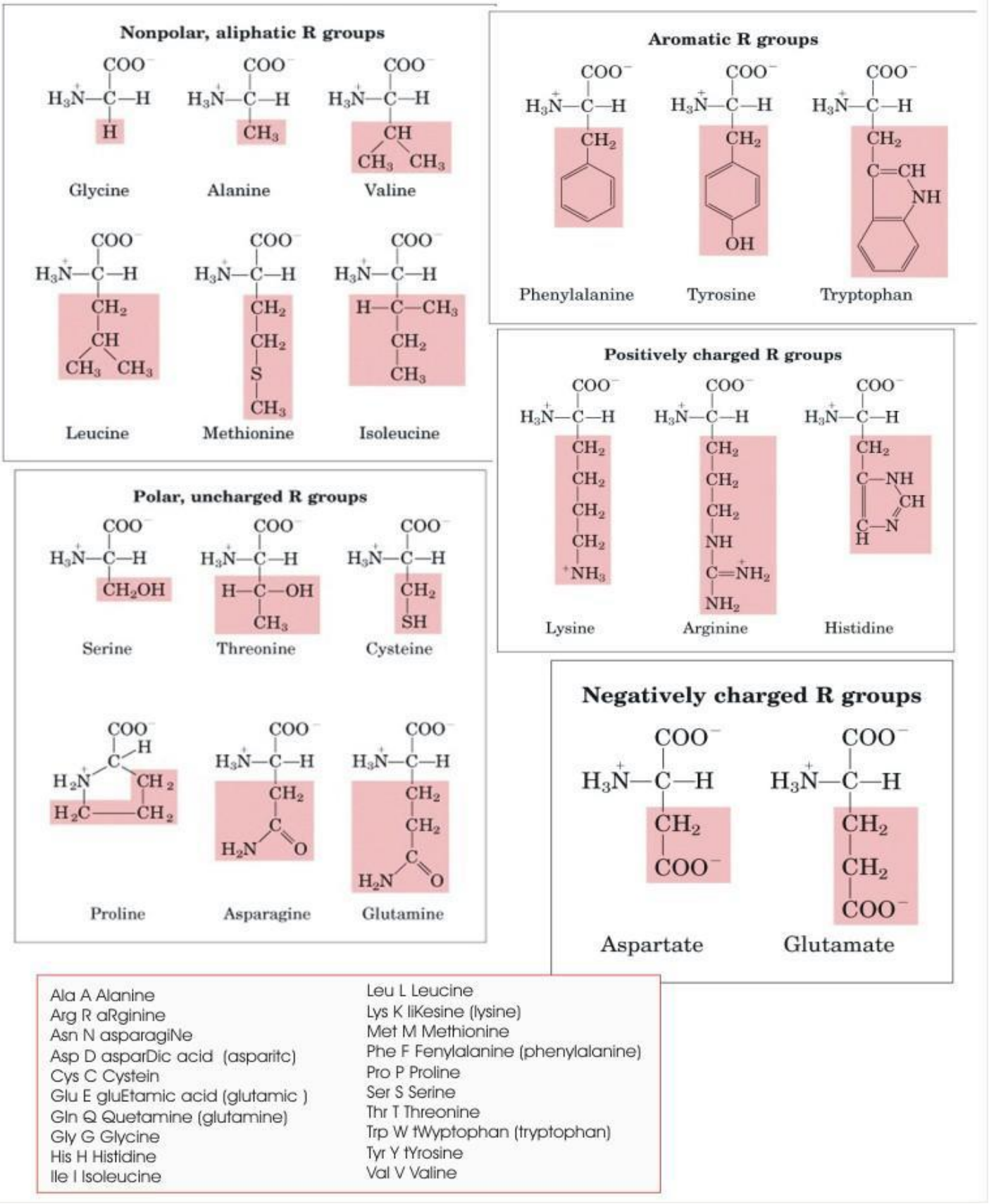


Fig 1. Structure of amino acids. The right-hand side figure shows the peptide bond.

Twenty standard Amino Acids



Ala A Alanine
Arg R aRginine
Asn N asparagiNe
Asp D asparDic acid (asparitc)
Cys C Cystein
Glu E gluEtamic acid (glutamic)
Gln Q Quetamine (glutamine)
Gly G Glycine
His H Histidine
Ile I Isoleucine
Leu L Leucine
Lys K liKesine (lysine)
Met M Methionine
Phe F Fenylalanine (phenylalanine)
Pro P Proline
Ser S Serine
Thr T Threonine
Trp W tWyptophan (tryptophan)
Tyr Y tYrosine
Val V Valine

which vary from one to another according to the type of their side chains, see Figure 2. Amino acids are amphoteric with an extremely high melting point (usually exceeding 200°C), react as amines in some reactions, but as carboxylic acids in others.

At a certain pH, the number of the positive charges (from protonated ammonium groups) are equal to the number of the negative charges (from deprotonated carboxylate groups), therefore, no net charge will be observed. This point is named as “the isoelectric point” and the amino acid will exist as zwitter ion. Herein, the Isoelectric point (Ip) is the pH value at which the concentration of anionic and cationic groups is equal (i.e. the net charge of this molecule equal to zero). It is the point where the molecule does not move to either the cathode or the anode when it is put in an electric field.

Because its solubility will be minimum, it is possible to precipitate at this point.

Amino acids are responded to all typical reactions associated with compounds containing carboxylic acid and amino groups, usually under conditions where the zwitter ions form is present in only small quantities. Amino acids are chiral molecules (except glycine) and exhibit an optical activity due to the presence of an asymmetric α-carbon atom.

Amino acids with an L-configuration are present in all naturally occurring proteins, whereas those with D-form are found in antibiotics and in bacterial cell walls.

Amino acids are classified into groups according to their side chains, see Figure 2.

The amino acids tests are:

1. Solubility test

The solubility of amino acids largely depends on the pH of the solution. Any change in the pH value causes structural changes in the amino acids and alters the relative solubility of the molecule.

In acidic solutions, both amino and carboxylic groups are protonated, whereas, in basic solutions, both groups are deprotonation.

Materials

Different amino acid solution: Gly, Lys and Glu.

Solvents: H_2O, HCl, NaOH and Chloroform.

Method

Mix a small amount of each amino acid solution with few mls of each solvent, record the results.

Questions?

1. What is the best solvent of amino acids? Why?
2. Can you dissolve Gly in water?
3. Can Lys be dissolved in NaOH?
4. What is the difference you can see when dissolve Lys and Glu in chloroform?
5. Do most amino acids dissolve in water?

2. Ninhydrin reaction

Ninhydrin [tri-ketohydrindene hydrate] is a powerful oxidizing agent used for detecting ammonia or primary and secondary amines.

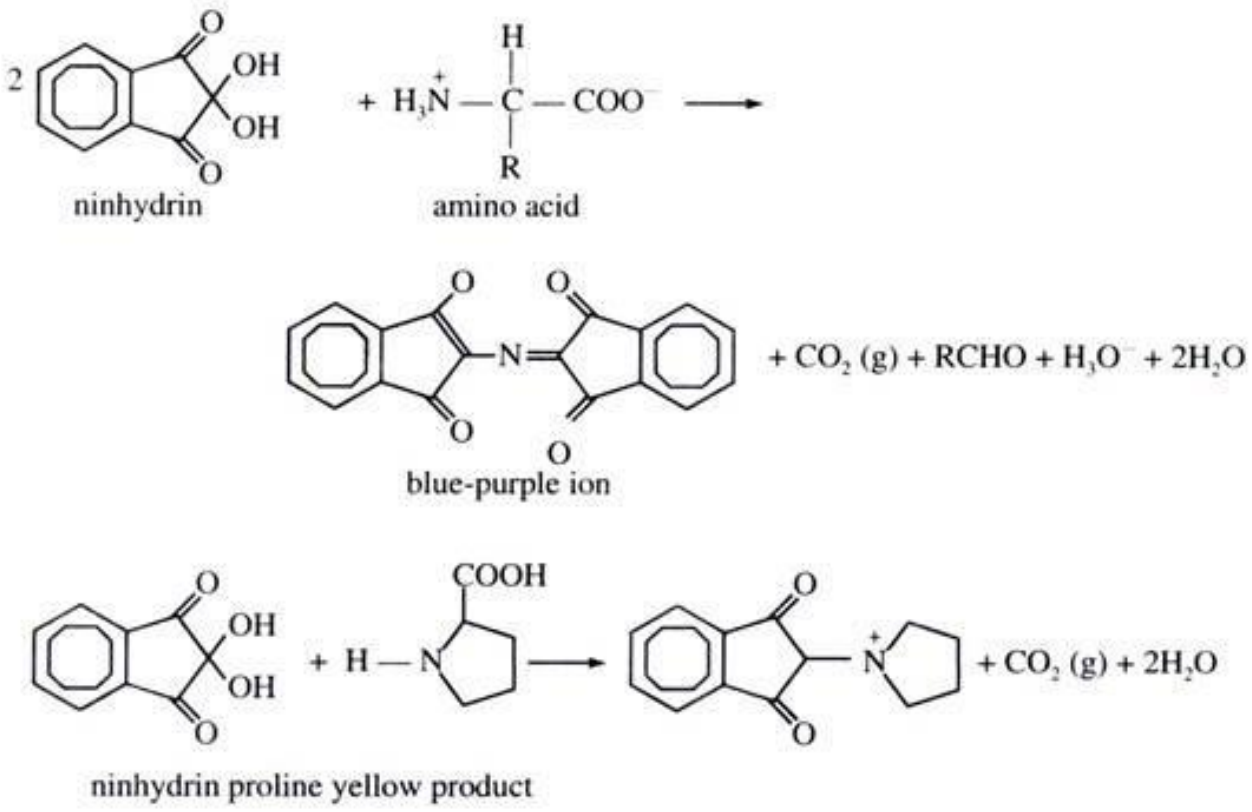


Fig 3. The ninhydrin coupling reaction with amino acids and with Pro.

In a range of pH around 4-8, all α-L-amino acids react with ninhydrin to give a purple colored product (diketohydrin). Ninhydrin

interacts with amino acids causes decarboxylation and deamination (liberation of CO_2 and ammonia) to produce the purple Rhuemann's compound and aldehyde, see Figure 3.

The amino acids Pro and hydroxyl Pro react with ninhydrine, but they give a yellow colored complex instead of a purple one.
Primary amines and ammonia react similarly, but without the liberation of CO_2. Different molecules such as peptides and proteins are also able to positively react with ninhydrin test.

Mechanically, one reduced ninhydrin molecule condenses with another non-reduced ninhydrin molecule and ammonia to form a violet –blue or purple condensation product, see Figure 4.

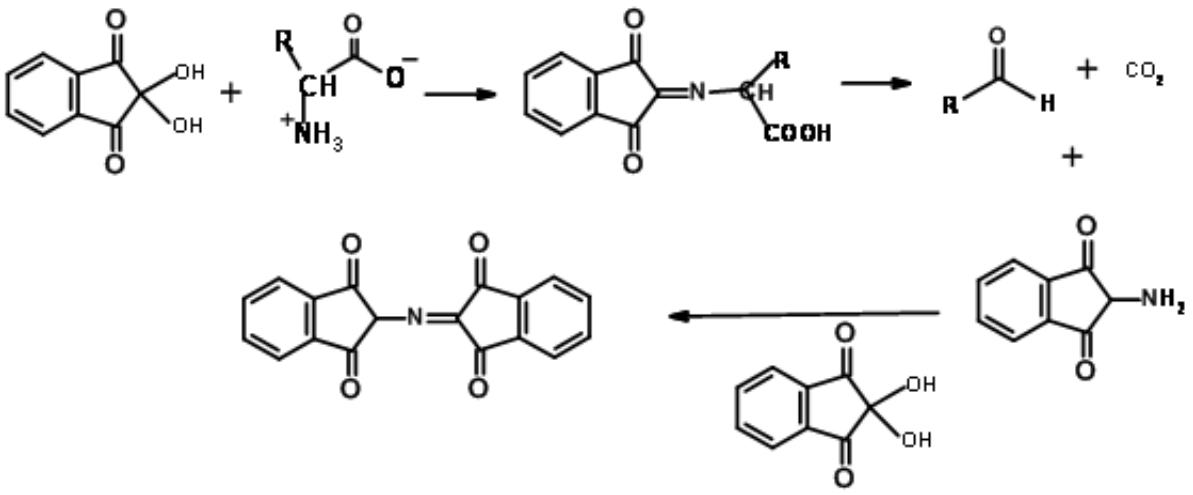


Fig 4. The mechanism of ninhydrin coupling reaction with amino acids.

Caution

Ninhydrin is a strong oxidizing agent; therefore, it should be handled with care. Apart from that, eyewear, gloves and hood are required. If accidentally get in touch with skin, extremely wash with water. However, the resulting stains are temporary and will be eliminated within 24 hours.

Method

1. Add 1ml of each of the test solutions to 2 drops of ninhydrin solution.
2. Boil the mixture over a water bath for 2 min.
3. Allow to cool and observe the resulted blue color.

Questions?

1. Which of the following gives a positive ninhydrin test?

Phospholipid, sucrose, galactose, Pro, Albumin.

2. What is ninhydrin reaction? How it is useful?
3. How dose methylamine (CH_3NH_2) reacts with ninhydrin test?
4. What kinds of protection you should use when doing ninhydrin experiment?
5. Why Pro and hydroxyl Pro give yellow color with ninhydrin?

3. Xanthoproteic reaction

This test is used to differentiate between aromatic amino acids. Treating aromatic amino acids with concentrated nitric acid leads to the nitration of the aromatic ring and formation of a yellow nitro-product (nitro derivatives), see Figure 5.

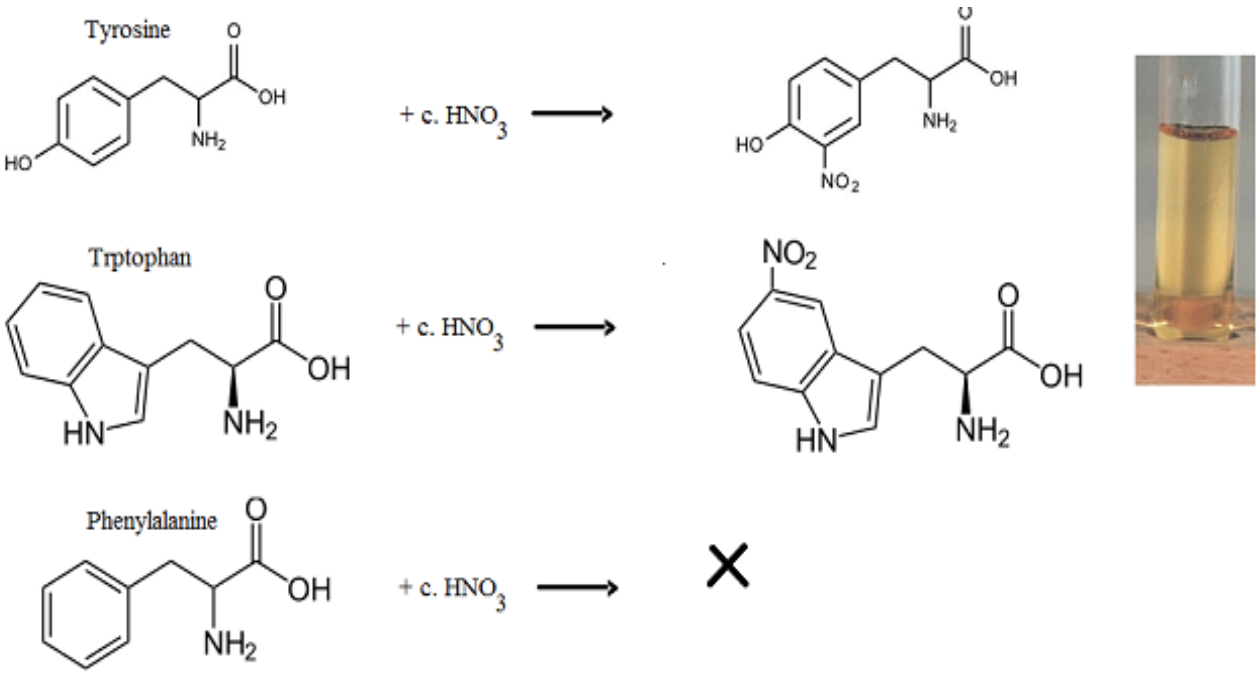


Fig 5. The Xanthoproteic reaction with aromatic amino acids

When a strongly basic solution is added, the color of the product turns to be darker (from

yellow to orange) due to the ionization of the petrolic group.

Caution

Concentrated HNO_3 is a toxic and corrosive substance that can cause severe burns and discolor the skin. Gloves, safety glasses and a fume hood are required. Avoid inhaling vapors and ingesting the compound.

Method

1. Add 1m of concentrated HNO_3 to 1ml of the test sample.
2. Heat the mixture and cool it.
3. Slowly add (40% w/v in a water solution) NaOH until the mixture becomes alkaline (use litmus strip to indicate the pH) and color-change is noted. Changing the color from yellow to orange indicates the presence of an aromatic amino acid.

Questions?

1. Which of the following gives positive test with Xanthoprotic reaction?
   Trp, Cys, Ala, Gly, Tyr, Thr
2. What is HNO_3 doing for the reaction?
3. what is the color of a positive reaction with Xanthoprotic?
4. Phe does not react with Xanthoprotic, why?

4. Millon`s reaction

The test was developed by the French chemist Auguste Million. Million`s reagent is a mixture of mercuric nitrate/nitric acid and water.

This test is specific for Tyr, the only amino acid contains a phenol group (a hydroxyl group attached to a benzene ring). However, all phenols give positive results with Million`s test.

The reaction occurs in 2 steps: first, the phenolic group of Tyr nitrates by HNO_3, then in the presence of $Hg(NO3)_2$, the nitrated Tyr forms a reddish-brown solution or a yellow precipitate of a nitrated Tyr, which is a positive test, Figure 6.

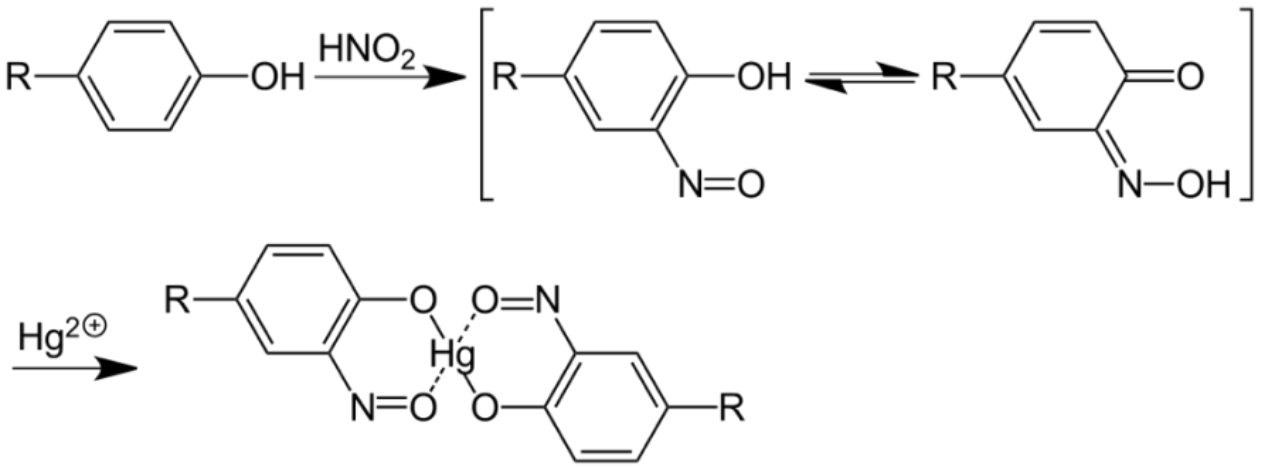


Fig 6. The Millon reaction with a phenolic amino acid.

Method

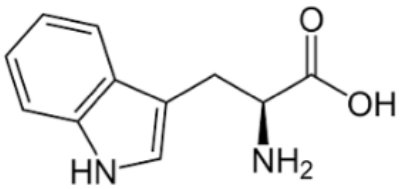
1. To 1ml of amino acid solution, add 1ml of Million`s reagent in a test tube
2. Warm the tube in a boiling water bath for 10 min.
3. A reddish-brown color is a positive reaction.

Questions?

1. Why Millon`s reagent gives a reddish color with phenolic amino acids?
2. Which test is more specific for Tyr? Millon`s or Xanthoprotic test? Why?
3. If you increase the concentration of Tyr, will that increase the depth of the product?
4. What Hg is doing for the reaction?
5. What is Millon reagent?

5. Acree-Rosenheim reaction

Acree-Rosenheim is another test for detecting Trp in a protein solution. Tryptophan is mixed with formaldehyde (CH_2O). Concentrated sulfuric acid is added to form two layers. A violet zone in the junction point of two layers is the positive result, see Figure 7. Similar result obtained by the Hopkins-Cole reaction which is frequently used to detect Trp in a protein solution. In the Hopkins-Cole reaction, when a glyoxylic acid CHOCOOH is added to a protein solution (containing Trp) in the presence of H_2SO_4, a violet ring will form.



+ H_2-C=O $\xrightarrow{H_2SO_4\ \text{Conc.}}$ a violet ring

Fig 7. Acree-Rosenheim reaction with Trp.

Method

(Acree-Rosenheim Reaction)

1. Add 1 ml of amino solution to 1ml of CH_2O.
2. Concentrated H_2SO_4 (1 ml) is then added to form two layers.
3. Watch the development of a purple colored ring between the two layers as positive test for Trp appearance.

Questions?

1. What is Acree-Rosenheim reagent?
2. What is the difference between Acree-Rosenheim and Hopkins-Cole reaction?
3. If the protein solution contains a mixture of Trp, phe and Tye, which amino acids form the purple ring more quickly?

4. How would the phenolic amino acid be distinguished by Acree-Rosenheim reaction?
5. If you have Phe, which of the following test is the best to detect?

Ninhydrine, Million, Xanthoproteic, Hopkins-Cole?

6. Pauly reaction

Pauly reaction was firstly described by the German chemist Hermann Pauly. It is used to detect Tyr or His amino acids by a coupling reaction with diazotized sulfanilic acid under

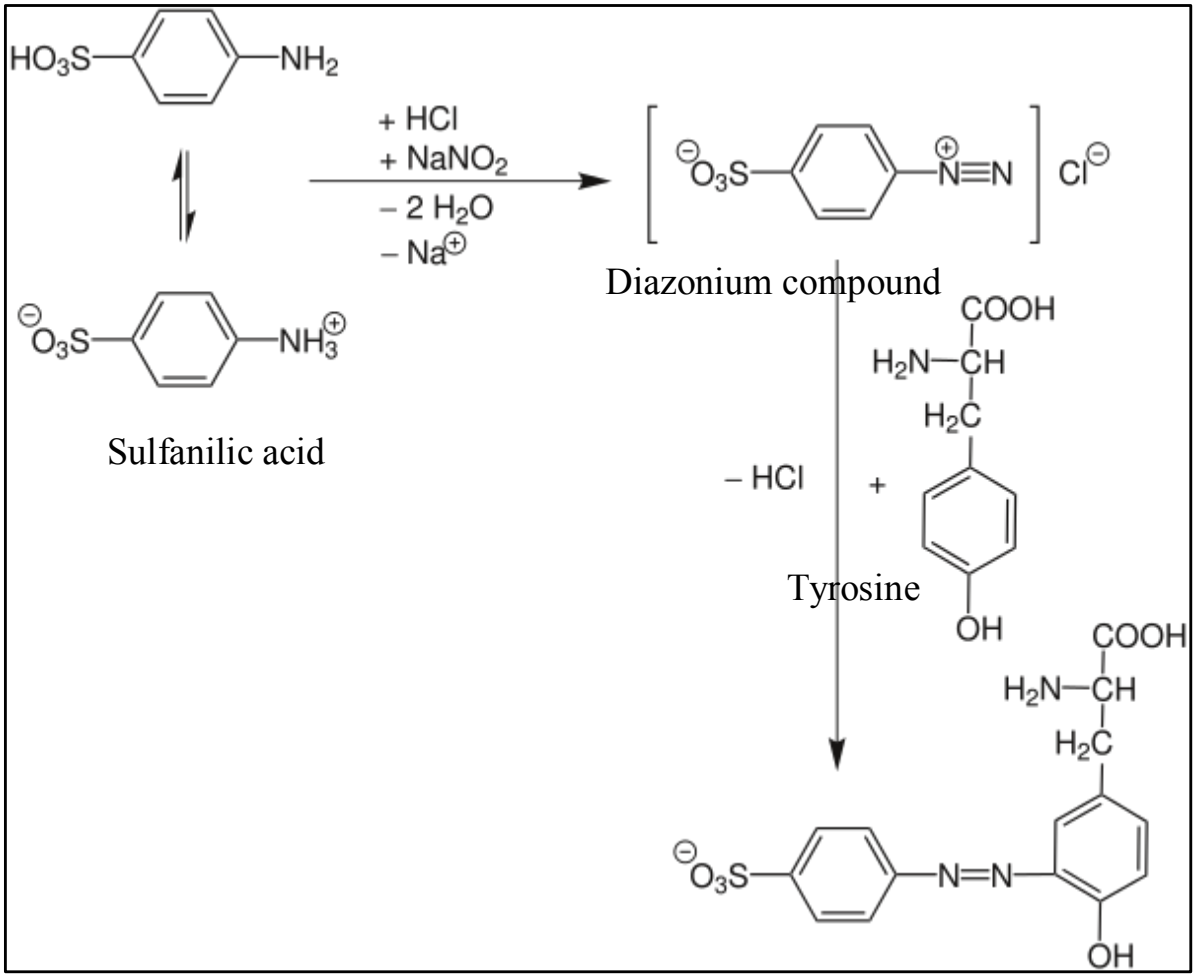


Fig 8. Pauly reaction with Tyr.

alkaline conditions. As a result, a red colored solution is produced.

Principally, diazotized sulfanilic acid is resulted by a reaction between the sulfanilic acid and sodium nitrite and sodium carbonate to form the diazonium component. Then, the diazonium components react with the imidazole ring of His or phenolic group of Tyr to form a dark red compound, see Figure 8.

Method

1. Cool down a test tube using a cool icebox.
2. Add 1ml of sulfanilic acid to the cool test tube, remain in the icebox.
3. Add to the mixture 1ml of $NaNO_3$, let it cool for 3min.
4. Add 2ml of Na CO_3.
5. Observe the color.

Questions?

1. How does the Pauly test work?
2. Which amino acid gives a positive product with Pauly reagent? and which gives negative product?
3. How does His react with Pauly?

7. Lead sulfide test

This is specific for sulfhydryl group (-SH). Sulphur containing amino acids, such as Cys and Cyst is converted into sodium sulfide (Na_2S) when boiled with 40 % NaOH. The reaction depends on a partial conversion of the organic sulfur into inorganic sulfide Na_2S, which can be detected using sodium plumbate ($Na_2pb(OH)_4$: lead acetate solution $Pb(CH_3COO)_2$ in an alkaline media). Na_2S precipitates as a black lead sulfide, see Figure 9. Methionine or any other Meth containing-proteins such as Casein and Gelatin, gives a negative result with this test.

$$\text{Protien-SH} + 2\,\text{NaOH} \xrightarrow{\Delta} \underset{\text{Na-Sulfide}}{\underset{40\%}{Na_2S}}$$

$$Na_2S + Pb(CH_3COO)_2 \xrightarrow{NaOH} PbS + 2\,CH_3COONa$$

Fig 9. Lead sulphide reaction with sulphur-containing amino acids.

Method

1. Prepare sodium plumbate ($Na_2Pb(OH)_4$ solution as following:

   Add 5ml of 40% NaOH to 2ml of dilute $Pb(CH_3COO)_2$. A white precipitate of Lead hydroxide $Pb(OH)_2$ will be formed.
2. Boil solution (1) to completely dissolve $Pb(OH)_2$ and form $Na_2Pb(OH)_4$.
3. Add few drops of 40% NaOH to 2ml of amino acid solution and boil the solution for 2min.
4. Cooldown solution (3) and add few drops of solution (2), then watch the development of a brown color or precipitate (PbS) as an indication of the (-SH) group.

Questions?

1. If you have a mixture of Cys, Cyst, Meth, Gly and Gln, do you expect the reaction will be positive with Lead sulfide test?

2. Why do you use sodium plumbate in lead sulfide teat?
3. How can you know that the Lead sulfide test is positive?
4. Can you use this test for most of amino acids?
5. How the Lead acetate test works?

8. Nitroprusside test

This test is specific for Cys which has a free sulfhydryl group (-SH) that is able to react with the nitroprusside in the presence of excess ammonia (NH_4OH).

Principally, the free thiol group of Cys gives a red color with sodium nitroprusside in the presence of NH_4OH, see Figure 10. To compare: do the test to Cys, Cystaein and Meth.

Nitroprusside test is commonly used to detect the ketones in urine.

Fig 10. Na-nitroprusside reaction with Cys amino acid.

Method

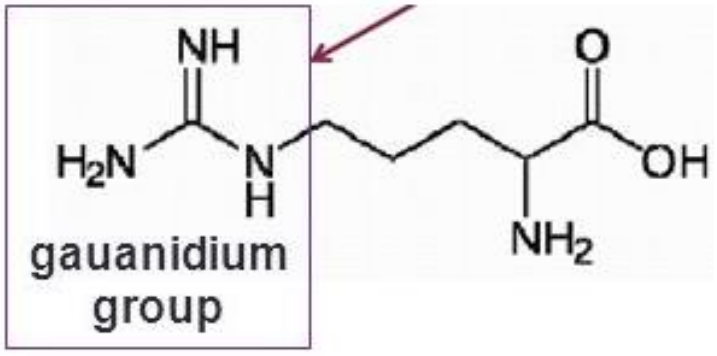
1. Add 0.5ml Na-nitroprusside solution to 2ml of amino acid solution and shack well.
2. Add 0.5ml NH_4OH and watch the change in the color.

Questions?

1. Do you think there is a difference between Lead acetate and Nitroprosside test? What is it?

9. Sakaguchi`test

Sakaguchi`s test is specific for the guanidine group-containing amino acids, such as Arg. Guanidine group interacts with α-naphthol and alkaline hypobromite to produce a red-colored complex.



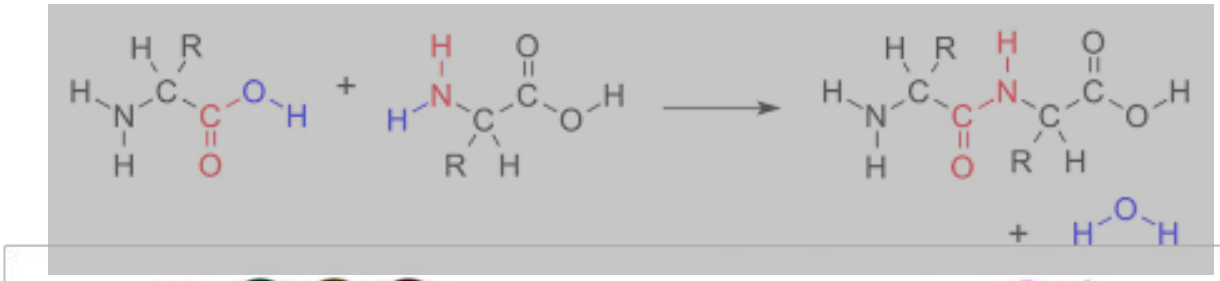
Method

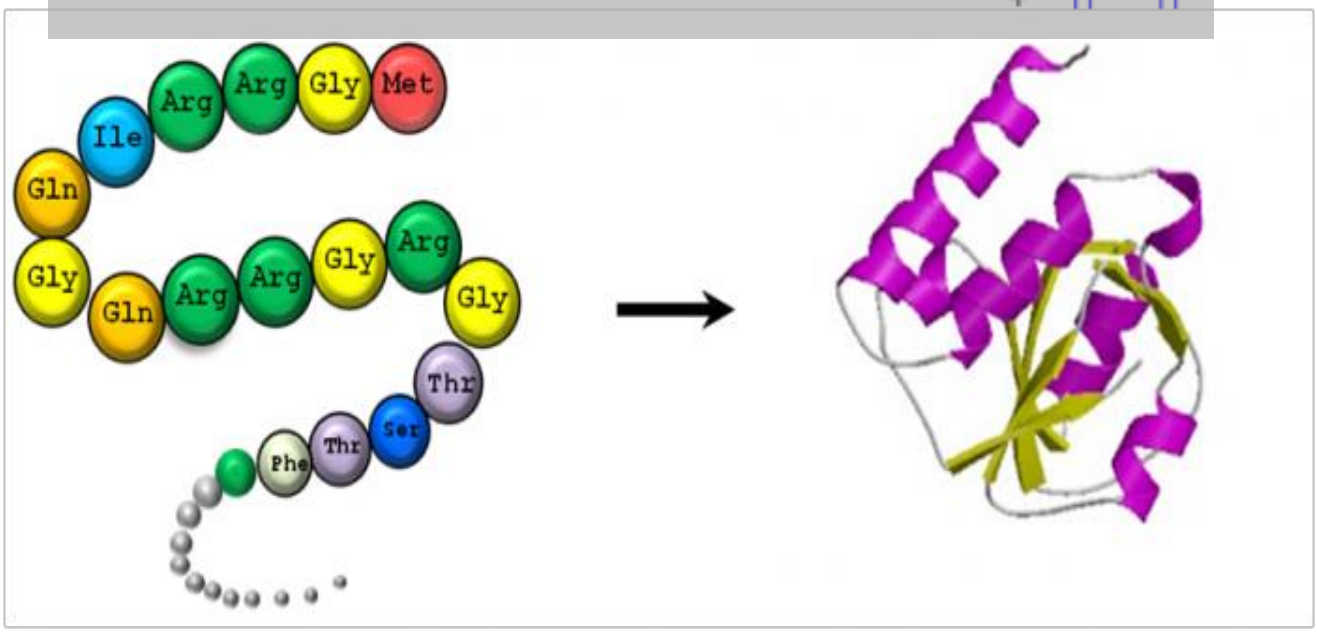
1. To 1ml of amino acid solution, add 2 ml of 40% NaOH and mix well.
2. Add 2ml of α-naphthol solution and mix well.
3. Add 2 drops of sodium hypobromite (BrNaO) solution, mix well and record the results (a red colored complex).

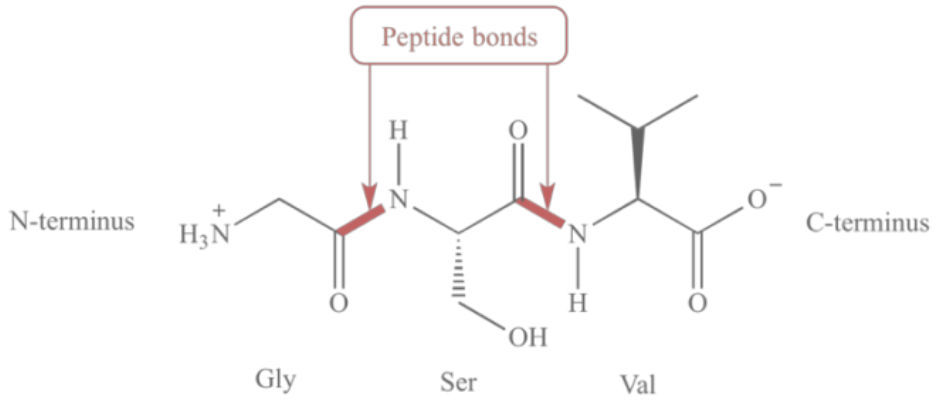
Questions?

1. What is the principle of Sakaguchi` test?
2. Why is this test specific for Arg?
3. What is the alkaline medium that you need for this test?
4. What is the positive result of this test?
5. What the will be result if used tertiary amine with Sakaguchi reagents?

Qualitative Tests of Proteins







Proteins

Proteins are polypeptides consist of many amino acids bound together via peptide bonds. Amino acids interact with each other via different forces to build up the final three-dimensional (3D) structure of the protein. These forces are responsible for the stability and rigidity of the protein, which are: van der Waals force between temporary dipoles, ionic interactions between charged groups, salt-bridges and polar-polar interactions.

Proteins regulate a variety of activities in all the known living organisms, ranging from the replication of a genetic code to transporting oxygen. Proteins exist as a 3D

tertiary and quaternary substance. The functional properties depend on the 3D structure, which arises when particular sequences of amino acids in a polypeptide chain fold to generate linear chains have compact domains with specific structures. These folded domains either serve as modules for larger polypeptide assemblies, or they support specific catalytic or binding sites for other functional substances or metal ions.

Here are the main tests for detecting the presence of a protein in a solution:

1. **Biuret test**

Biuret test is used to indicate the presence of peptide bonds within the protein molecule. This test could be useful to quantitatively know the concentration of the protein, where the intensity of the product`s color increases

linearly with the concentration of the protein. In addition, according to the Beer-Lambert law, the optical density at 540 nm wavelength is directly proportionated to the protein concentration.

Biuret reagent (H_2N-CO-NH-CO-NH_2) results from a coupling reaction of two urea molecules. It reduces Cu(II) to Cu (I) and

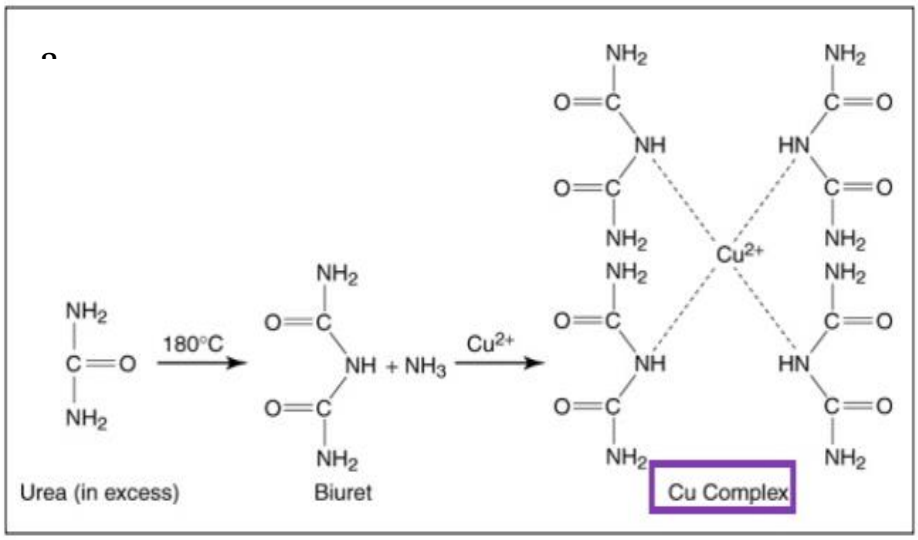


Fig 11. a) Reaction of Biuret with Cu(II), b) interaction of Biuret reagent with the peptide bond.

form a red colored Cu-complex as a positive result for C=O-NH bond involvement, see Figure 11a.

Because Biuret has a bond (C=O-NH) similar to the peptide bond, it is the most useful test for samples with two peptide bonds and above, see Figure 11b. Buffers, such as Tris and ammonia interfere with this assay.

Method

1. Add 1 ml of 40% NaOH to 1 ml protein sample to make it alkaline.
2. Add 5 ml Biuret reagent to solution (1)
3. Watch changing the color within 5 min.

Questions?

1. If you let your sample to stand 20 min with Biuret Reagent, what will happen?
2. What is the component of Biuret reagent?
3. What is the principle of Biuret reaction?
4. Which of the following do you expect will give aa positive result with Biuret? Glucose? Casein? Glu? Phospholipid?
5. Why the reagent called Biuret?.

2. Precipitation reactions

a. Denaturation of proteins

Denaturation is an irrevocable change in the protein structure lead to loss of its biological properties. Because the fold type of the protein (β-sheet, α-helix, random coil) determines its function, any change of the tertiary structure will alter its activity. Temperature and pH are the main causes of denaturation and precipitation of proteins because they destroy the weak bonds and affect the tertiary and secondary, but not the primary structure.

Method

1. In a water bath, boil 1ml protein of different protein solutions such as Bovine albumin and egg globulin.
2. Record your results

b. Precipitation by heavy metals

At natural pH 7 or above, proteins are usually negatively charged. Adding positively charged metal ions will neutralize the charges of protein and lead to protein precipitation. Heavy metals such as Ag^{+}, Pb^{2+}, Hg^{2+}, etc form a complex with the alkaline proteins and then precipitate.
NOTE: avoid very high pH to reduce the risk of metal hydroxides precipitation.

Method

1. To 2 ml of different protein solutions (e.g., Bovine albumin, egg white, gelatin, casein), add few drops of: $AgNO_3$, Lead acetate $Pb(CH_3COO)_2$. to each protein solution.
2. Observe the extent of precipitate in each experiment.

c. Precipitation by organic acids

In acidic medium, proteins are positively charged. Adding acidic solutions such as picric acid and Trichloroacetic acid (TCA) will neutralize the protein charge and irreversibly form an insoluble solution due to their high content of negative charges.

Method

1. To 2 ml of protein solution (albumin solution or egg white solution), add 5-8 drops of picric acid or TCA solution.
2. Note the precipitate in each protein tube.
3. Slowly add (1 ml) of 40% NaOH solution and record if increasing the pH will cause renaturation of the protein (dissolve the precipitate).

d. Precipitation by neutral salts

To investigate the effect of salt concentrations on the protein solubility, a series of low concentrations of salt is added to a protein solution (salting in). low concentrations will increase the protein solubility. Salt molecules decrease the electrostatic energy between the protein molecules, which increase the solubility and stability of the protein structure.

Increasing the salt concentration (salting out) will decrease the protein solubility at some points. The excess of salt ions (not bound to the protein) competes with protein in binding the solvent molecules. This decrease in salvation allows the proteins to aggregate and precipitate. Each protein can be reversibly precipitated at specific salt concentration.

This test is used to separate different proteins using high salt concentration solution (oversaturation solutions), which will precipitate the certain protein due to the effect of salt ions on the protein solubility. Proteins precipitate at different salt concentrations; therefore, a series of salt concentrations are required to test the precipitation ability of each protein.

In salting out the experiment, you must take into account the following:

- The type of the salt (ammonium sulfate $(NH_4)_2SO_4$ is commonly used)
- The molecular weight (Mwt) of the protein: the highest Mwt, the fastest to precipitate.
- Proteins with high Mwt usually require the low concentration of salt to precipitate, and vice versa.

- It is a reversible reaction; the protein can be recovered by dissolving with H_2O
- Applied the salting out experiment on a mixture of protein

Method (salting in)

1. To 2 ml of albumin solution (egg white) add (1ml) NaCl solution.
2. Add an equivalent amount of $(NH_4)_2SO_4$ until you notice over saturation.
3. Repeat the experiment with other proteins such as globulin, mucin and lysozomes. Describe the difference in the solubility or precipitation ability.

Method (salting out)

1. Add (1ml) globulin solution in (1ml) 0.1 M NaCl.

2. Add 1ml globulin solution to 1ml saturated $(NH_4)_2SO_4$ and record your observation.
3. Add 1ml egg albumin to 1ml saturated $(NH_4)_2SO_4$ and record your observation. Add excess amount of solid $(NH_4)_2SO_4$ to indicate the oversaturation.

e. Denaturation by alcohol

Some alcoholic reagents such as ethanol or propanol can disrupt the hydrogen bonding within the protein structure by intra hydrogen-bonding with the protein molecules. For sterilizing purposes, 70% alcohol is used to denature the protein of the bacteria because it effectively penetrates the bacterial cell wall. Whereas, 95% alcohol has no similar effect, probably because it coagulates the surface proteins as a crust, which would prevent the alcohol to penetrate into the cell wall.

Method

1. Treat 2ml of a protein solution with a few drops of alcohol.
2. Notice the protein precipitation.

f. Denaturation by acids (Heller`s test)

Nitric acid desaturates proteins and form a white precipitate.

Method

1. Add 1ml HNO_3 (conc). to 1ml of protein solution.
2. Note the protein precipitate formed.

g. Precipitation by Esbach`s reactions

It is a mixture of dilute acids (picric acid, citric acid and water) used to quantitatively detects albumin in the urine. This reagent precipitates protein where a yellowish to brown color product is formed depending on the quantity of the protein.

Method

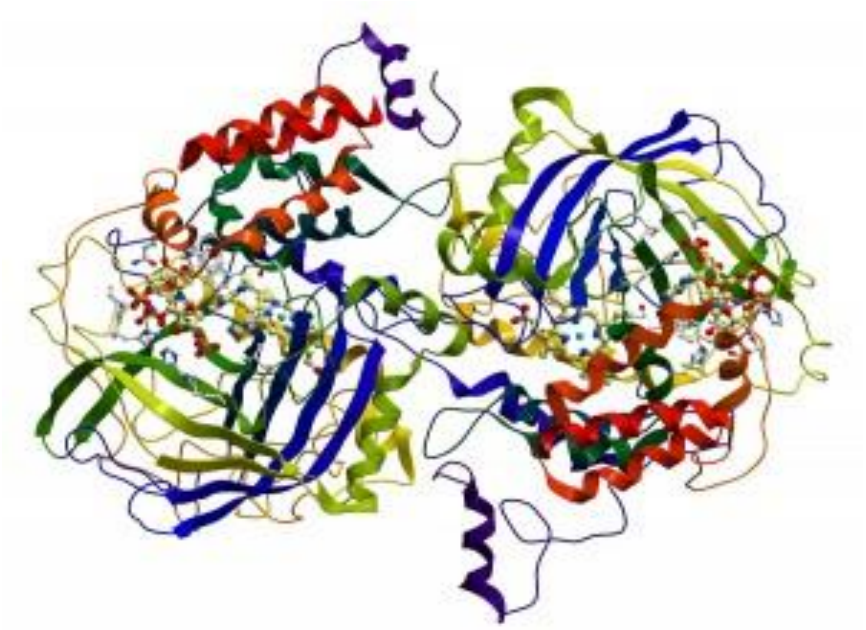
1. Add 1ml Esbach reagent to 1ml of protein solution.
2. Note the color of protein precipitate.

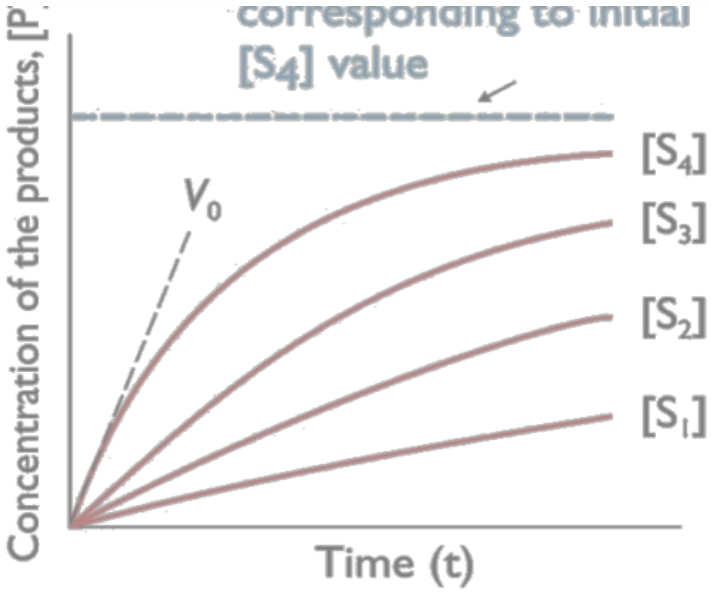
Questions?

1. What causes denaturation of protein?
2. If you heat the protein solution up to 70 C, will that affected the tertiary structure of the protein? The secondary structure?

3. Why is $(NH_4)_2SO_4$ precipitated protein?
4. What is slating-out protein? Salting in protein?
5. Which of the following metals causes precipitation of the protein? Why?
   Hg, Al, Ni, Ca, Zn, Cu, Ag, Au, Pb, Na.
6. What is the difference between organic acid and normal acid in terms of precipitation the protein? Which one causes reversible precipitation?
7. Do you think that absolute ethanol is better than 70% ethanol to precipitate albumin?
8. What is Heller reagent?
9. Do you think that adding HNO_3 will cause reversible denaturation?
10. what is the best test to distinguish Albumin? Globulin?

Enzyme kinetics





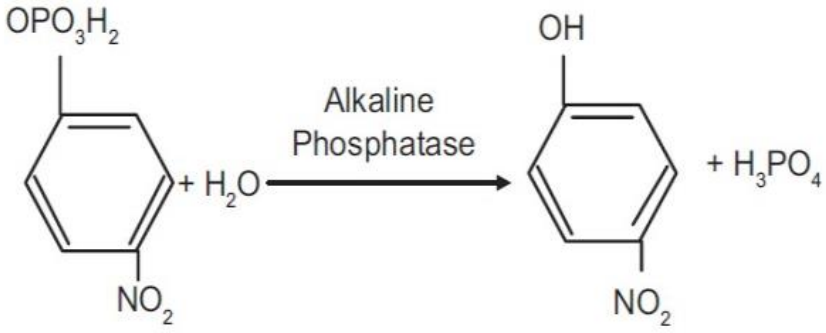
Enzymes

The Enzyme is a biological molecule with an active site to bind a substrate and accelerate a chemical reaction.

Determination of enzyme kinetics

The aim of this practice is to experimentally obtain data for the kinetics of an enzymatic reaction in the presence and absence of an inhibitor. This data is then will be used to produce Lineweaver-Burke plots in the presence and absence of an inhibitor to determine K_m, V_{max} and the type of inhibition.

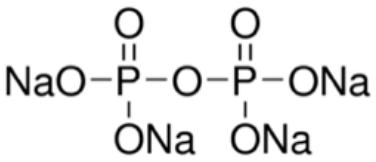
In this experiment, the alkaline phosphatase enzyme (ALP) catalyzes the hydrolyses of the substrate *p*-nitrophenyl phosphate (*p*-NPP) in alkaline medium to produce *p*-nitrophenol (*p*-NP) and phosphoric acid, as following



p-nitrophenyl phosphatephosphate *p*-nitrophenol

The product p-NP is yellow under the alkaline pH of the assay. Therefore, the rate of the enzyme-catalyzed reaction can be followed by recording the absorption readings at 405nm of the amount of product accumulated as a function of time (min).

In the second part of the experiment, you will determine the type of inhibition when Na-pyrophosphate is added to the enzyme reaction mixture.



Tetra sodium pyrophosphate

Reagents

Substrate: 0.02 M *p*-NPP (stock solution)
Buffer: 0. L M-glycine/NaOH buffer pH 10.4
Enzyme: ALP (2 mg/ml in 0.1M-glycine/NaOH buffer pH 10.4)
Inhibitor: 100 mM Na-pyrophosphate or 200 mM imidazole

Method

Using clean plastic cuvettes, set up reaction mixtures as instructed in Table 1. Set the spectrophotometer up using buffer as a blank (no enzyme and no substrate). Set the blank to zero at 405nm. Leave the blank inside the instrument for the rest of the experiment.
Now start the enzyme assays. Do all the addition to the cuvette and leave the enzyme solution **LAST**. Cover the cuvette by a parafilm and mix by inversion. Remove the

parafilm, put the cuvette into the spectrophotometer and read the absorbance (Abs) at 1 min. intervals for 6 min.

Repeat tube 1 but without adding the enzyme, can you record a rate for the chemical reaction?

Table 1. The reaction mixture of enzyme kinetics without inhibitor I.

Tube no.	Buffer (ml)	Substrate (ml) 0.02 M p-NPP	Na-PP (I) ml 100 mM	H_2O (ml)	Enzyme solution(ml) **LAST**
Blank	1.9	-	-	1.3	-
1	1.9	1.0	-	0.2	0.1
2	1.9	0.8	-	0.4	0.1
3	1.9	0.6	-	0.6	0.1
4	1.9	0.4	-	0.8	0.1
5	1.9	0.2	-	1.0	0.1
6	1.9	0.1	-	1.1	0.1
Non-Enzyme rate	1.9	1.0	-	0.3	None

1) Calculation of substrate concentration [S]

Calculate the substrate concentration [S] from the concentration of the stock solution of p-NPP (0.02M) and its dilution in each of the enzyme reaction mixtures (1-6) using the dilution low $N_1V_1=N_2V_2$.

2) Calculation of the enzyme velocity V_0

The initial velocity V_0 is the amount of the product per a unit of time (min.) and NOT ITS
CONCENTRATION. Plot the absorbance reading (Abs) of each enzyme reaction against time and derive the V_0 of the reaction from the slope as Abs /min.
Convert each Abs/min to mol *p*-NP (the product/min) using the extinction coefficient

(ε) of *p*-NP (15 $mM^{-1}cm^{-1}$) and the volume of the assay mixture (3.2 ml) for each tube, as following:

Abs= ε * l * C (Beer-Lambert law)

$$C = \frac{Abs.min^{-1}}{15\ mM^{-1}\ \cancel{cm^{-1}} * 1\ \cancel{cm}}$$

$$C = Abs.min^{-1}\frac{1}{15} * \frac{mmol}{mL}$$

$$C = \frac{Abs.min^{-1}}{15} * \frac{mmol}{\frac{1000}{3.2}}$$

$$C = \frac{Abs.min^{-1}}{15} * \frac{3.2}{1000}\ \mu mol$$

$$C = Abs.min^{-1} * 0.21\ \mu mol$$

$= ?\ \mu mol.min^{-1}$

(Dividing by 15 converts the Abs into concentration (mM) units; as a M solution is the gram Mwt (mol) in 1000 ml). The volume of the assay is 3.2 ml, the amount of product is determined by multiplying (Abs/min.15) by the fraction 3.2/1000, assuming that all [S] is dissociated to product.

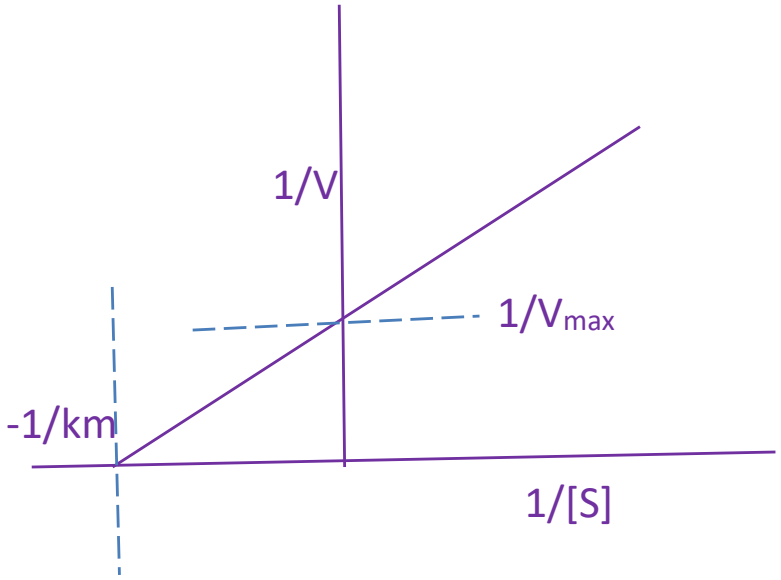
(i) V_0 against [S]

Plot V_0 for each enzyme-reaction (µmol/min) against each [S] (mM) to obtain the V max.

(ii) Lineweaver-Burke plot to determine Km and V max

Calculate the reciprocals of each of the values of V_0 (1/V) and of [S] and plot $1/V_0$ against l/[S]. Draw the straight line of best fit. This line intercepts the l/V axis at l/Vmax and the 1/[S] axis at -l/Km as in the example below:

$$\frac{1}{V} = \frac{1}{Vmax} + \frac{Km}{Vmax}[S]$$



3) Determination of the type of inhibition

Repeat the experiment in the Table 1 using 0.2 ml of Na- pyrophosphate in each cuvette as instructed in Table 2. (* Note the volumes of water is differ than that in Table 1).

Table 2. The reaction mixture of the enzyme kinetics with inhibitor I.

Tube no.	Buffer (ml)	Substrate 0.02 M p-NPP	Na-PP (I) ml 100 mM	H_2O (ml)	Enzyme solution LAST
Blank	1.9	-	0.2	1.1	-
1	1.9	1.0	0.2	-	0.1
2	1.9	0.8	0.2	0.2	0.1
3	1.9	0.6	0.2	0.4	0.1
4	1.9	0.4	0.2	0.6	0.1
5	1.9	0.2	0.2	0.8	0.1
6	1.9	1.0	0.2	0.9	0.1

(i) V_0 against [S] in the presence of I

Plot V_0 for each enzyme-reaction (µmol/min) against each [S] (mM) as used in a and i.

(ii) Lineweaver-Burke plot in the presence of [I]

Calculate the reciprocals of each of the values of V_0 and of [S] as appeared in Table 3 and plot $1/V_0$ against l/[S] as you used for **(ii)**. Draw the straight line of best fit and indicate the type of the inhibition in terms of Km and V max.

Table 3. Table of the reciprocals values of V_o and S in the presence and absence of inhibitor I					
[S]	1/[S]	V (μmole/min)	1/V	V_I (inhibitor)	$1/V_I$
?	?	?	?	?	?
?	?	?	?	?	?
?	?	?	?	?	?
?	?	?	?	?	?
?	?	?	?	?	?
?	?	?	?	?	?
?	?	?	?	?	?

Questions?

1. Can you ignore the mixture of tube 1 for the non-enzymatic rate? Why?
2. From your plot, what do you conclude regarding the effect of increasing [S] on the velocity?
3. What are factors influencing the initial velocity V_o?
4. Is the effect of the inhibitor that you observed representative of

competitive? Non-competitive inhibition? Why?

5. What is the K_i of the inhibitor?

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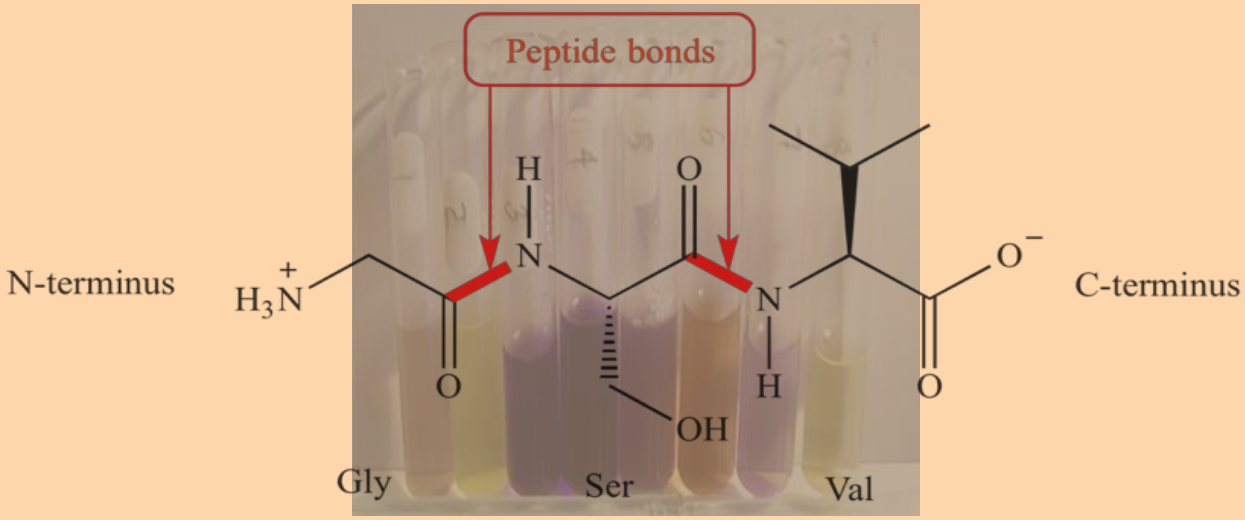

Hotspot on the author

Dr. Zahraa S. Al-Garawi is a prof assist in Biochemistry and a PhD in Biophysics, Structural biology and Nanomaterials from University of Sussex, UK. She previously has wrtten a practical handbook in Clinical Biochemistry for undergraduates, in two languages, in Arabic and English. Zahraa has published very high-quality works in her field. Recently, she published a scientific textbook about steps for understanding writing and publishing papers, for researcher and student"

Zahraa and Aliaa collaborate in this handbook to provide the most trusted methods for amino acid and protein tests, and the enzyme kinetics. This handbook would be of good benefits and helpful for undergraduates, chemistry and biology department.