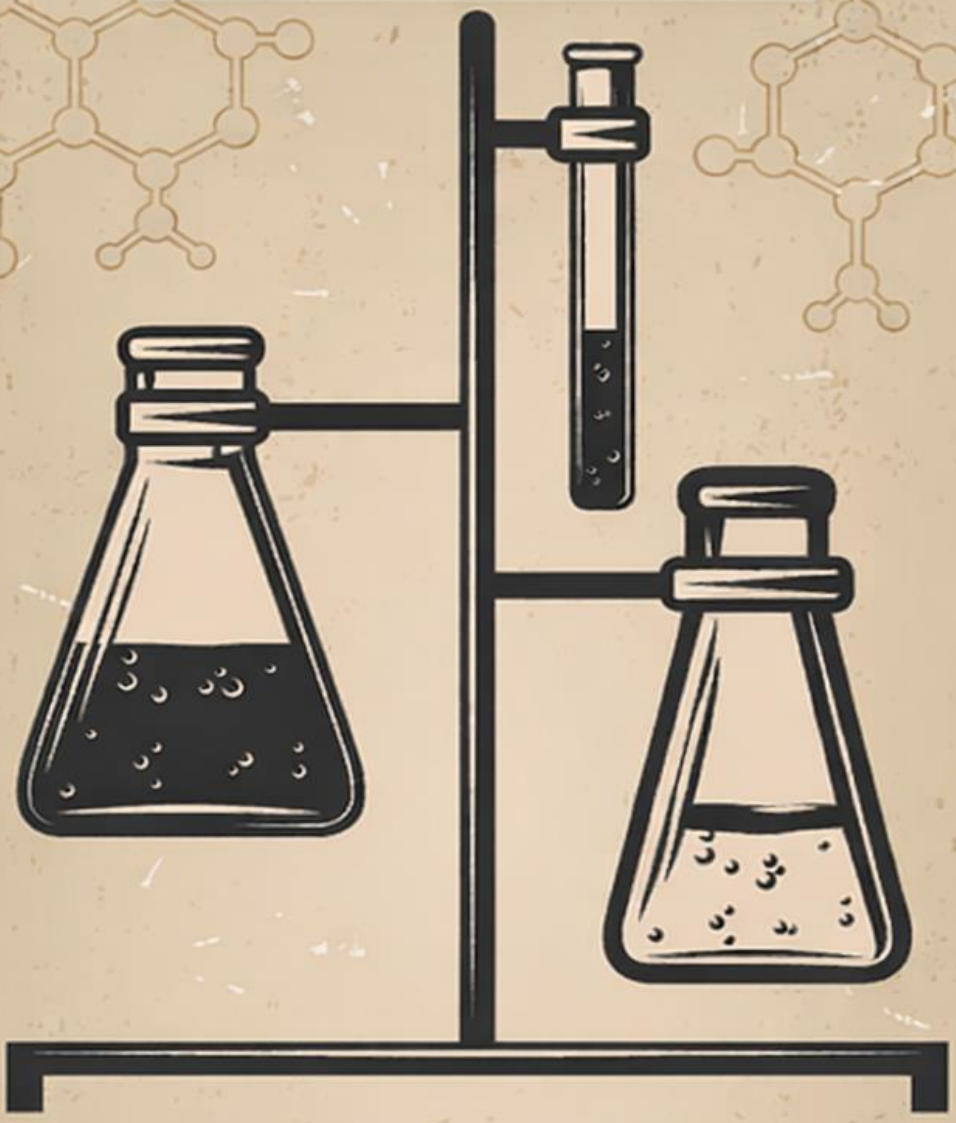
Laboratory research

Facult of Science
Department of Chemistry



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SCIENCE AND TECHNOLOGY

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Laboratuvar Kuralları ve Güvenlik

Her laboratuvardan önce o gün yapılacak olan deneyi mutlaka dikkatle okuyunuz. Her deneyden önce bir kısa sınav yapılacaktır. Bu sınav sonucunda sıfır almış olan öğrenci o deneye alınmaz.

Laboratuvarda çalışırken laboratuvar önlüğü ve (steril olmayan) laboratuvar eldiveni kullanılır. Önlük ve eldivenlerinizi laboratuvardan ayrılıncaya kadar çıkarmayınız. Bazı yumuşak lensler laboratuvar kimyasalları ile tepkimeye girerek rahatsızlık oluşturacağından, eğer mümkünse kontak lenslerinizi laboratuvara girmeden önce çıkarınız. Deney esnasında asistanların talimatlarını dikkatle uygulayınız. Deney bitiminde tüm cihazları kapatıp, çalıştığınız yerin temizliğini iyi bir şekilde yapınız.

Bazı kimyasallar zehirli, mutajenik, karsinojenik veya teratojenikdir (doğum kusurlarına yol açan). Bu kimyasalların kullanımında ve atılmalarında laboratuvardaki görevlilerin yaptığı uyarıları dikkate alınız.

Tüm öğrencilerin bir laboratuvar not defteri tutmaları istenmektedir. Deney esnasında yaptığınız her bir ölçümü, deney aşamasını, dikkatle laboratuvar not defterinize not alınız. Her deneyin tamamlanmasından sonra en geç bir sonraki deney günü deney raporlarınızı teslim ediniz. Bu raporlar https://kmy-fen.marmara.edu.tr/ogrencilere/biyokimya-anabilim-dali-duyurular web sayfasında bulunan Rapor Formatına göre hazırlanmalıdır.

Öğrenciler deneyleri gruplar halinde yapmaktadırlar, ancak her bir öğrenci kendi yorumlarını, kendi hazırladığı deney raporu ile sunmalıdır.

Birbirinin aynı olan ve/veya çalışma sorularına cevap verilmemiş deney raporları ile yapay zekanın doğrudan kullanımının (>%20) tespit edildiği raporlar kabul edilmeyecektir.

Her dönemde bir ara sınav yapılır. Laboratuvar kısa sınavlarının %10'u deney raporlarının %20'si ve ara sınavın %70'i alınarak öğrencinin vize notu saptanır. Ara sınavdan sonra yapılan deneyler için de laboratuvar kısa sınavlarının %10'u deney raporlarının %20'si ve sınavın %70'i alınarak öğrencinin final notu saptanır.

Her deneyden önce yapılan kısa sınava girmeyen ya da sınavda başarısız olan öğrenci o deneye giremez ve girmediği deneyin raporunu veremez.

EXPERIMENT I

Determination of Antioxidant Effects of Various Spices

Reactive oxygen species are the main free radicals formed in many redox processes and frequently cause oxidative damage to biomolecules such as carbohydrates, proteins, lipids, and DNA. Degeneration of biomolecules and subsequent initiation of oxidative chain reactions cause accelerated aging and many chronic diseases such as neurodegenerative diseases, cancer, and cardiovascular diseases. Antioxidant compounds play important roles in the prevention and treatment of such diseases by scavenging free radicals and preventing the oxidation of biomolecules. Nowadays, many studies are being carried out on natural antioxidants that have many pharmacological activities such as anticarcinogenic, antimutagenic and neuroprotective.

Different methods are used to measure the antioxidant properties of a substance. Different methods are used to measure the antioxidant properties of a substance. These methods are: FRAP (ferric reducing antioxidant power), ORAC (Oxygen Radical Absorbance Capacity) and, most commonly, the examination of the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging effect. The DPPH radical (DPPH·) is purple in color due to its unpaired nitrogen atom and when it reacts with the oxygen atom of a radical scavenger, the yellow reduced DPPH-H (2,2-diphenyl-1-picrylhydrazine) is formed. This color change is followed spectrophotometrically at a wavelength of 517 nm and thus the antioxidant power of any substance is calculated (Figure 1).

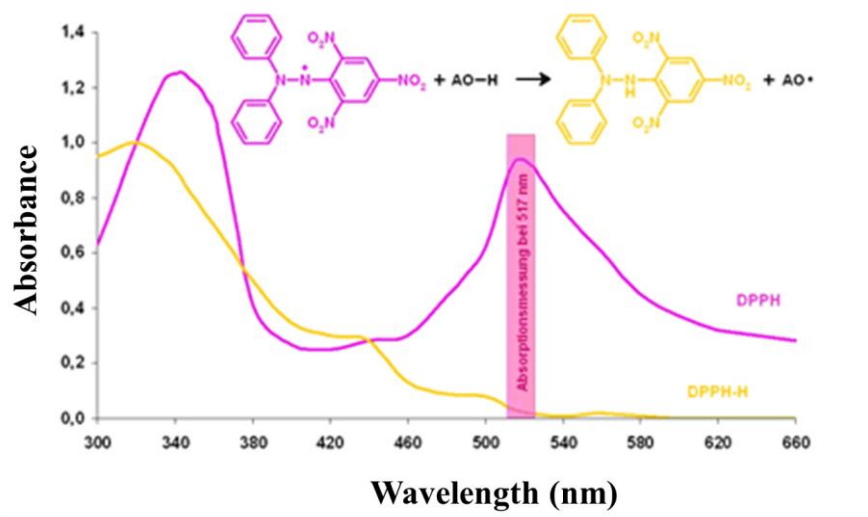


Figure 1. Spectra of DPPH and DPPH-H in UV-Vis.

METHOD

Chemicals and Solutions

The purpose of this experiment is to determine the antioxidant power of ethanolic extracts from various plant sources. **For this purpose, each group will bring a different plant sample or spice to the experiment (An edible sample is preferred)**. 1 g of each sample will be weighed. Therefore, it is important to avoid bringing an excessive number of samples.

0.1 mM DPPH solution: Prepared by dissolving 0.0038 g DPPH in 100 mL ethanol. The solution should be stored in the refrigerator and in the dark when not in use. It should be used within 7-8 hours after preparation.

Take 0.5 g of sample from each experimental group and then make small portions. If grinding is required, add 10 mL of ethanol after placing the sample in a mortar or beaker and process the sample continuously for at least 2 min with a glass rod (or pestle). Using a Pasteur pipette, collect the liquid portions in a clean graduated tube and record the approximate volume. If particles are present in the solution, centrifugate at 4000 g for 5 minutes.

PROCESS

The sample is diluted with ethanol at various ratios (total volume as 0.6 mL sample and 1 mL ethanol) (1/10, 1/50, 1/100 etc.) and 1 mL of DPPH solution is added. After the tube is thoroughly vortexed, it is waited for 10 minutes, and its absorbance is measured against ethanol at a wavelength of 517 nm (Table 1). These are the $\mathbf{A_1}$ values.

1 mL of DPPH is added to 1.6 mL of ethanol and measured against ethanol at a wavelength of 517 nm after 10 minutes. This is the $\mathbf{A_0}$ value.

Table 1. Dilution and measurement method.

Sample (mL)	Methanol (mL)
0.1	0.9 (1/10)
0.1	4.9 (1/50)
0.1	9.9 (1/100)
Take 0.6 mL of the dilution solutions and then add 1 mL of ethanol	
Add 1 mL of DPPH solution Wait 10 minutes in the dark	
Absorbance measured at 517 nm	

DATA USE

The DPPH% scavenging effect of different dilutions of each sample is calculated according to the following formula:

$$\text{DPPH scavenging effect\%} = [(A_0-A_1)/A_0] \times 100$$

The DPPH% scavenging effect graph is then plotted against concentration to calculate the concentration required to scavenge 50% of the DPPH radical. This value is known as the **EC_{50}** value (the concentration of the substance required to exhibit half of its maximum effect) and is used to compare extracts from different sources. After determining the graph equation, 50 is assigned to the y-value, and calculations are performed to determine the EC_{50} value, for example.

QUESTIONS

1- What are the health benefits and harms of antioxidant consumption?

2- Find a scientific article published last year about antioxidants and briefly write what methods were used and what results were found.

EXPERIMENT II

Quantitative Ascorbic Acid Analysis

Ascorbic acid (vitamin C) is a hexose derivative with an enediol structure and a lactone ring (Figure 1). It is water-soluble, colorless, odorless and crystalline. It is stored for long periods in dry conditions in places where there is no light. Many animals and plants synthesize ascorbic acid, but for some mammals and humans, vitamin C is essential and must be taken in the diet at a rate of approximately 75 mg per day. Ascorbic acid, which is abundant in fresh vegetables and fruits, is a strong reducing agent and plays an important role in delaying aging and preventing cancer, as well as acting as an antioxidant in the body. It has been suggested by Linus Pauling that taking very high doses of vitamin C, which is widely used in the treatment and prevention of colds, protects against cancer. However, recent studies have suggested that consuming excessive amounts of vitamin C may trigger cancer. In addition, scurvy disease, which manifests itself as capillary bleeding, gum infections and tooth decay, occurs in the absence of vitamin C. This disease is rarely seen today because of the addition of synthetic ascorbic acid to soft drinks. Excess vitamin C is excreted in the urine.

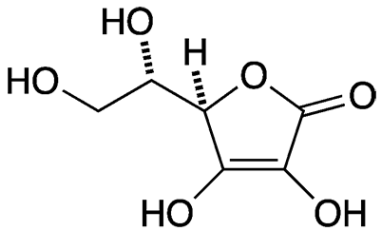


Figure 1. Structure of ascorbic acid.

Ascorbic acid is stable in acidic solutions. Ascorbic acid, which has a strong reducing effect, decomposes and loses its effect when heated. The determination of ascorbic acid in foodstuffs, drugs and natural products is based on this reducing property of the compound. Titrimetric techniques are the main analytical methods used in determination. Titrations with iodine and 2,6-dichlorophenol-indophenol, fluorometric procedures, photochemical reactions with methylene blue are among these.

METHOD

The purpose of this experiment is to determine the amount of ascorbic acid in various commercial or conventional fruit juices as well as different kinds of drink. Therefore, **each group will bring diverse beverages.**

Chemicals and Solutions

0.25% starch solution: 0.625 g starch and dissolved in 250 mL of hot water, boiled until the solution becomes clear.

0.7 M sodium thiosulfate solution: 0.1 g Na_2CO_3 and 11.08 g $Na_2S_2O_3$/1000 mL water.

0.2% KIO_3 solution: 2 g KIO_3/1000 mL water.

5% KI solution: 2.5 g/50 mL water (prepared separately for each group).

0.1% Ascorbic acid standard solution: 0.1 g ascorbic acid/100 mL water.

0.3 M H_2SO_4 solution: 16.65 mL concentrated H_2SO_4/1000 mL water.

PROCESS

Erlen No	H_2SO_4 (mL)	Ascorbic acid (mL)	Beverage (mL)	Water (mL)	KIO_3 (mL)	10 minutes of incubation in the dark.	KI (mL)	Titration with $Na_2S_2O_3$ (Until light yellow)	Starch (mL)	Titration with $Na_2S_2O_3$ (Until colorless)	Consumption
1	50	2.5	0	17.5	15		10		2		
2	50	10	0	10	15		10		2		
3	50	20	0	0	15		10		2		
Sample	50	0	20	0	15		10		2		

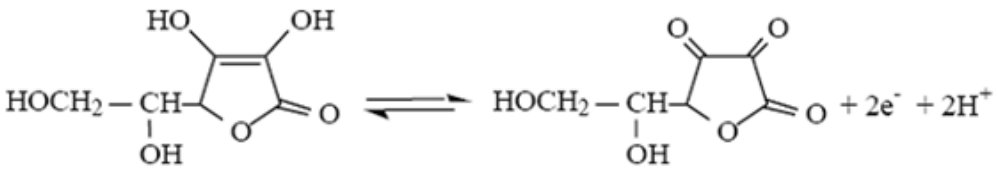
Ascorbic acid is a medium strength reducing agent. The reaction of reducing the iodine molecule in water with ascorbic acid is given as follows;

- $KIO_{3(aq)} + 6\ H^+_{(aq)} + 5\ I^-_{(aq)} \rightarrow 3\ I_{2(aq)} + 3\ H_2O_{(l)} + K^+_{(aq)}$ (I_2 formation)
- $C_6H_8O_{6(aq)} + I_{2(aq)} \rightarrow C_6H_6O_{6(aq)} + 2\ I^-_{(aq)} + 2\ H^+_{(aq)}$ (Oxidation of vitamin C)

I_2 is formed by reaction 1 and this I_2 is oxidized by reaction 2. Both reactions occur in dilute acidic conditions and reaction 1 requires I^- ions.

The relevant half-reactions are given as follows;

- $I_2 + 2e^- \rightarrow 2\ I^-$



The total reactions are as follows;

- Ascorbic acid + $I_{2(aq)}$ + H_2O → Dehydroascorbic acid + $2I^-$ + $2H^+$

The equilibrium constant of this reaction is high, and the reactants are completely converted to products.

- $I_{2(aq)} + I^- \rightarrow I_3^-$

However, I_2 has low solubility in water. Therefore, I^- is used to form the I_3^- complex. The I_3^- complex is called triiodide. Triiodide can also be produced using iodate.

- $IO_3^- + 8\ I^- + 6\ H^+ \rightarrow 3\ I_3^- + 3\ H_2O$

Triiodide can also be produced using iodate.

- Ascorbic acid + I_3^- + H_2O → Dehydroascorbic acid + $3I^- + 2H^+$

Triiodide reacts with ascorbic acid.

- $I_3^- + 2\ S_2O_3^{2-} \rightarrow 3$ I- + $S_4O_6^{2-}$

The concentration of ascorbic acid is determined indirectly by determining the remaining unreacted I^{3-} ions. Thiosulfate is used for this purpose. $S_4O_6^{2-}$ is called thionate ion. Starch is used as an indicator. Triiodide forms a dark blue complex with starch.

DATA USE

The amount of ascorbic acid in Erlenmeyer flasks 1, 2 and 3 is calculated in mg and plotted against the thiosulfate consumption. The ascorbic acid concentration is determined by calculating the consumption of the sample on the graph and is expressed as mg/100mL.

QUESTIONS

1- Which organisms synthesize ascorbic acid through which metabolic pathways?

2- Briefly explain the structure of collagen. What changes occur in the structure of collagen in the absence of ascorbic acid?

EXPERIMENT III

Buffer Solutions

Solutions that contain a weak acid together with its conjugate base (anion), or a weak base together with its conjugate acid (cation), are referred to as buffer solutions. Buffer solutions resist significant changes in pH upon the addition of small amounts of acid or base. For a solution to exhibit buffering capacity, it must contain sufficient concentrations of both proton-donating (H^+-releasing) and proton-accepting species.

Buffers are prepared to function within specific pH ranges. Their ability to maintain a relatively constant pH is critical in many chemical and biological systems.

The Henderson–Hasselbalch equation is used to calculate the theoretical pH of a buffer solution. As indicated by the equation, altering the ratio of the conjugate base (A^-) to the weak acid (HA) allows preparation of buffer solutions with different pH values.

$$pH = pK_a + \log\frac{[A^-]}{[HA]}$$ **Henderson-Hasselbalch Equation** (A^-: Salt / HA: Acid)

Buffer components do not exhibit optimal buffering capacity at all ratios. Maximum buffering occurs when the ratio A^-/HA equals 1. Since log(1) = 0, the pH equals the pKa value under these conditions. This pH is referred to as the optimum pH of the buffer. Buffer effectiveness decreases as the pH moves away from this value. In practice, buffers are most effective within ±1 pH unit of their pKa.

Buffers are essential in the preparation and maintenance of biological environments. In living organisms, pH values are maintained within very narrow ranges and are tightly regulated. Even small deviations in pH affect the charged groups of enzymes, cell membranes, nucleic acids, and other biomolecules, thereby altering their physiological functions.

A change of approximately 0.5 pH units may threaten the viability of an organism. Different physiological compartments exhibit distinct pH values, for example:

- Blood: 7.4
- Gastric juice: 1.5
- Pancreatic fluid: 8.0
- Cerebrospinal fluid: 7.4

These pH values are maintained by various buffer systems, including:

- Bicarbonate / Carbonic acid (HCO_3^- / H_2CO_3)
- Protein / Proteinate systems
- Oxyhemoglobin / Protonated oxyhemoglobin (HbO_2 / $HHbO_2$)
- Phosphate buffer system (HPO_4^{2-} / $H_2PO_4^-$)

A shift of body fluids toward the acidic side is termed acidosis, whereas a shift toward alkalinity is termed alkalosis. If acidic metabolic products increase, the generated H^+ ions are buffered by the bicarbonate/CO_2 system, forming carbonic acid (H_2CO_3). Dissociation of carbonic acid produces additional H^+ ions, which are subsequently buffered by other systems. Some H_2CO_3 is converted into carbon dioxide and water. An increase in CO_2 concentration lowers the pH below 7.4. When this occurs due to a decrease in HCO_3^- concentration, the condition is referred to as metabolic acidosis. Examples include acidosis observed in diabetes, starvation, and carbohydrate-restricted diets. Conversely, an increase in HCO_3^- concentration results in metabolic alkalosis, which may occur due to excessive sodium bicarbonate intake or loss of gastric HCl during severe vomiting.

CHEMICALS

Monopotassium phosphate (KH_2PO_4)

Disodium phosphate (Na_2HPO_4)

Sodium hydroxide (NaOH)

Hydrochloric acid (HCl)

METHOD

Using standard buffer preparation tables, prepare:

50 mL of pH 7.4 phosphate buffer (Sørensen buffer)

25 mL of pH 6 citrate/phosphate buffer

QUESTIONS

1- A buffer solution contains 0.01 mol lactic acid (pKa = 3.86) and 0.05 mol sodium lactate per liter.

a) Calculate the pH of the buffer.

b) What will be the change in pH if 5 mL of 0.5 M HCl is added to 1 L of this buffer?

2- Which organs play the most significant roles in regulating pH in body fluids, and through which physiological mechanisms do they maintain acid–base balance?

No.	Name	pH range	Temperature	pH change per °C
	General buffers			
1	KCl/HCl (CLARK and LUBS)[1]	1.0- 2.2	Room	0
2	Glycine/HCl (SØRENSEN)[2]	1.2- 3.4	Room	0
3	Na citrate/HCl (SØRENSEN)[2]	1.2- 5.0	Room	0
4	K biphthalate/HCl (CLARK and LUBS)[1]	2.4- 4.0	20° C	+0.001
5	K biphthalate/NaOH (Clark and LUBS)[1]	4.2- 6.2	20° C	
6	Na citrate/NaOH (SØRENSEN)[2]	5.2- 6.6	20° C	+0.004
7	Phosphate (SØRENSEN)[2]	5.0- 8.0	20° C	−0.003
8	Barbital-Na/HCl (MICHAELIS)[3]	7.0- 9.0	18° C	
9	Na borate/HCl (SØRENSEN)[2]	7.8- 9.2	20° C	−0.005
10	Glycine/NaOH (SØRENSEN)[2]	8.6-12.8	20° C	−0.025
11	Na borate/NaOH (SØRENSEN)[2]	9.4-10.6	20° C	−0.01
	Universal buffers			
12	Citric acid/phosphate (MCILVAINE)[4]	2.2- 7.8	21° C	
13	Citrate-phosphate-borate/HCl (TEORELL and STENHAGEN)[5]	2.0-12.0	20° C	
14	BRITTON-ROBINSON[6]	2.6-11.8	25° C	at low pH 0 at high pH −0.02
	Buffers for biological media			
15	Acetate (WALPOLE)[7-9]	3.8- 5.6	25° C	
16	Dimethylglutaric acid/NaOH[10]	3.2- 7.6	21° C	
17	Piperazine/HCl[11,12]	4.6- 6.4 8.8-10.6	20° C	
18	Tetraethylethylenediamine*[12]	5.0- 6.8 8.2-10.0	20° C	
19	Trismaleate[7,13]	5.2- 8.6	23° C	
20	Dimethylaminoethylamine*[12]	5.6- 7.4 8.6-10.4	20° C	
21	Imidazole/HCl[14]	6.2- 7.8	25° C	
22	Triethanolamine/HCl[15]	7.0- 8.8	25° C	
23	*N*-Dimethylaminoleucylglycine/NaOH[16]	7.0- 8.8	23° C	−0.015
24	Tris/HCl[7]	7.2- 9.0	23° C	−0.02
25	2-Amino-2-methylpropane-1,3-diol/HCl[7,13]	7.8-10.0	23° C	
26	Carbonate (DELORY and KING)[7,17]	9.2-10.8	20° C	

Buffer No.	Stock solutions		Composition of the buffer
	A	B	
1	KCl 0.2-N (14.91 g/l)	HCl 0.2-N	25 ml A + x ml B made up to 100 ml
2	Glycine 0.1-molar in NaCl 0.1-N (7.507 g glycine + 5.844 g NaCl/l)	HCl 0.1-N	x ml A + (100 − x) ml B
3	Disodium citrate 0.1-molar (21.01 g $C_6H_8O_7 \cdot 1H_2O$ + 200 ml NaOH 1-N per litre)	HCl 0.1-N	x ml A + (100 − x) ml B
4	Potassium biphthalate 0.1-molar (20.42 g $KHC_8H_4O_4$/l)	HCl 0.1-N	50 ml A + x ml B made up to 100 ml
5	As No. 4	NaOH 0.1-N	50 ml A + x ml B made up to 100 ml
6	As No. 3	NaOH 0.1-N	x ml A + (100 − x) ml B
7	Monopotassium phosphate 1/15-molar (9.073 g KH_2PO_4/l)	Disodium phosphate 1/15-molar (11.87 g $Na_2HPO_4 \cdot 2H_2O$/l)	x ml A + (100 − x) ml B
8	Barbital sodium 0.1-molar (20.62 g/l)	HCl 0.1-N	x ml A + (100 − x) ml B
9	Boric acid, half-neutralized, 0.2-molar (corr. to 0.05-molar borax: 12.37 g boric acid + 100 ml NaOH 1-N per litre)	HCl 0.1-N	x ml A + (100 − x) ml B
10	As No. 2	NaOH 0.1-N	x ml A + (100 − x) ml B
11	As No. 9	NaOH 0.1-N	x ml A + (100 − x) ml B
12	Citric acid 0.1-molar (21.01 g $C_6H_8O_7 \cdot 1H_2O$/l)	Disodium phosphate 0.2-molar (35.60 g $Na_2HPO_4 \cdot 2H_2O$/l)	x ml A + (100 − x) ml B
13	To citric acid and phosphoric acid solutions (ca. 100 ml), each equivalent to 100 ml NaOH 1-N, add 3.54 cryst. orthoboric acid and 343 ml NaOH 1-N, and make up the mixture to 1 litre	HCl 0.1-N	20 ml A + x ml B made up to 100 ml
14	Citric acid, monopotassium phosphate, barbital, boric acid, all 0.02857-molar (6.004 g $C_6H_8O_7 \cdot 1H_2O$, 3.888 g KH_2PO_4, 5.263 g barbital, 1.767 g H_3BO_3/l)	NaOH 0.2-N	100 ml A + x ml B
15	Sodium acetate 0.1-N (8.204 g $C_2H_3O_2Na$ or 13.61 g $C_2H_3O_2Na \cdot 3H_2O$/l)	Acetic acid 0.1-N (6.005 g/l)	x ml A + (100 − x) ml B
16	ββ-Dimethylglutaric acid 0.1-molar (16.02 g/l)	NaOH 0.2-N	(a) 100 ml A + x ml B made up to 1000 ml (b) 100 ml A + x ml B + 5.844 g NaCl made up to 1000 ml (NaCl ≙ 0.1-molar)
17	Piperazine 1-molar (86.14 g/l)	HCl 0.1-N	5 ml A + x ml B made up to 100 ml
18	Tetraethylethylenediamine 1-molar (172.32 g/l)	HCl 0.1-N	5 ml A + x ml B made up to 100 ml
19	Tris acid maleate 0.2-molar (24.23 g tris[hydroxymethyl]-aminomethane + 23.21 g maleic acid or 19.61 g maleic anhydride/l)	NaOH 0.2-N	25 ml A + x ml B made up to 100 ml
20	Dimethylaminoethylamine 1-molar (88 g/l)	HCl 0.1-N	5 ml A + x ml B made up to 100 ml
21	Imidazole 0.2-molar (13.62 g/l)	HCl 0.1-N	25 ml A + x ml B made up to 100 ml
22	Triethanolamine 0.5-molar (76.11 g/l) containing 20 g/l ethylenediaminetetraacetic acid disodium salt ($C_{10}H_{14}O_8N_2Na_2 \cdot 2H_2O$)	HCl 0.05-N	10 ml A + x ml B made up to 100 ml
23	*N*-Dimethylaminoleucylglycine 0.1-molar (24.33 g $C_{10}H_{20}O_3N_2 \cdot \frac{1}{2}H_2O$/l) containing NaCl 0.2-N (11.69 g/l)	NaOH 1-N 100 ml made up to 1 litre with A	x ml A + (100 − x) ml B
24	Tris 0.2-molar (24.23 g tris[hydroxymethyl]aminomethane/l)	HCl 0.1-N	25 ml A + x ml B made up to 100 ml
25	2-Amino-2-methylpropane-1,3-diol 0.1-molar (10.51 g/l)	HCl 0.1-N	50 ml A + x ml B made up to 100 ml
26	Sodium carbonate anhydrous 0.1-molar (10.60 g/l)	Sodium bicarbonate 0.1-molar (8.401 g/l)	x ml A + (100 − x) ml B

pH	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16a	16b	17	18	19	20	21	22	23	24	25	26	pH
1.0	54.2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	1.0
1.2	36.0	11.1	9.0	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	1.2
1.4	23.2	26.4	17.9	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	1.4
1.6	14.7	36.2	23.6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	1.6
1.8	9.3	43.9	27.6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	1.8
2.0	5.9	50.7	30.2	...	...	...	...	...	...	...	...	...	74.4	...	...	...	...	...	...	...	...	...	...	...	...	...	...	2.0
2.2	3.8	56.5	32.2	...	...	...	...	...	...	...	...	98.8	68.8	...	...	...	...	...	...	...	...	...	...	...	...	...	...	2.2
2.4	...	62.3	34.1	41.0	...	...	...	...	...	...	...	94.5	64.6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	2.4
2.6	...	68.4	36.0	34.3	...	...	...	...	...	...	...	90.0	61.3	1.6	...	...	...	...	...	...	...	...	...	...	...	...	...	2.6
2.8	...	74.7	37.9	27.8	...	...	...	...	...	...	...	85.1	58.9	3.6	...	...	...	...	...	...	...	...	...	...	...	...	...	2.8
3.0	...	81.0	39.9	21.6	...	...	...	...	...	...	...	80.3	56.9	5.7	...	...	...	...	...	...	...	...	...	...	...	...	...	3.0
3.2	...	86.2	42.1	15.9	...	...	...	...	...	...	...	76.0	55.2	7.8	...	7.0	14.4	...	...	...	...	...	...	...	...	...	...	3.2
3.4	...	90.3	44.8	10.9	...	...	...	...	...	...	...	72.0	53.9	9.9	...	13.3	20.9	...	...	...	...	...	...	...	...	...	...	3.4
3.6	...	...	47.8	6.7	...	...	...	...	...	...	...	68.4	52.9	11.7	...	20.7	26.8	...	...	...	...	...	...	...	...	...	...	3.6
3.8	...	...	51.2	3.3	...	...	...	...	...	...	...	65.1	51.8	13.5	10.9	26.3	32.4	...	...	...	...	...	...	...	...	...	...	3.8
4.0	...	...	55.1	0.0	...	...	...	...	...	...	...	62.0	50.7	15.3	16.6	32.4	36.6	...	...	...	...	...	...	...	...	...	...	4.0
4.2	...	...	60.0	...	3.0	...	...	...	...	...	...	59.1	49.7	17.5	23.9	36.2	40.3	...	...	...	...	...	...	...	...	...	...	4.2
4.4	...	...	66.4	...	6.7	...	...	...	...	...	...	56.4	48.6	19.7	33.5	39.3	43.1	...	...	...	...	...	...	...	...	...	...	4.4
4.6	...	...	74.9	...	11.1	...	...	...	...	...	...	53.7	47.5	21.9	44.9	41.3	45.7	94.3	...	...	...	...	...	...	...	...	...	4.6
4.8	...	...	85.6	...	16.5	...	...	...	...	...	...	51.2	46.4	24.1	56.6	43.5	48.3	91.5	...	...	...	...	...	...	...	...	...	4.8
5.0	...	...	100.0	...	22.6	...	99.2	...	...	...	...	49.0	45.4	26.3	67.8	45.7	51.5	87.8	94.3	...	...	...	...	...	...	...	...	5.0
5.2	...	...	...	...	28.8	87.1	98.4	...	...	...	...	46.9	44.3	28.6	76.8	48.4	53.6	83.6	91.5	3.2	...	...	...	...	...	...	...	5.2
5.4	...	...	...	...	34.4	78.0	97.3	...	...	...	...	44.7	43.2	31.0	84.0	51.3	58.2	77.6	87.8	5.0	...	...	...	...	...	...	...	5.4
5.6	...	...	...	...	39.1	70.3	95.5	...	...	...	...	42.4	42.0	33.4	89.3	55.0	63.6	71.8	83.1	7.3	94.3	...	...	...	...	...	...	5.6
5.8	...	...	...	...	42.4	64.5	92.8	...	...	...	...	40.0	40.8	35.8	...	58.8	68.7	66.5	77.6	9.7	91.7	...	...	...	...	...	...	5.8
6.0	...	...	...	...	45.0	60.3	88.9	...	...	...	...	37.4	39.7	38.3	...	63.9	73.6	61.8	71.7	12.4	88.0	...	...	...	...	...	...	6.0
6.2	...	...	...	...	46.7	57.2	83.0	...	...	...	...	34.5	38.4	40.8	...	69.5	78.5	58.2	66.4	15.2	83.3	43.4	...	...	...	...	...	6.2
6.4	...	...	...	...	...	54.8	75.4	...	...	...	...	31.4	37.0	43.3	...	74.1	83.3	55.5	61.7	17.9	77.9	40.4	...	...	...	...	...	6.4
6.6	...	...	...	...	...	53.2	65.3	...	...	...	...	27.9	35.6	45.8	...	83.5	87.4	...	58.0	20.8	72.0	36.5	...	...	...	...	...	6.6
6.8	...	...	...	...	...	...	53.4	...	...	...	...	23.5	34.2	48.3	...	87.4	91.0	...	55.3	22.2	66.6	31.4	86.2	86.4	...	...	...	6.8
7.0	...	...	...	...	...	...	41.3	53.3	...	...	...	19.0	32.9	50.9	...	90.0	93.2	...	...	23.7	61.9	25.4	79.6	80.6	44.7	...	...	7.0
7.2	...	...	...	...	...	...	29.6	55.0	...	...	...	13.8	31.7	53.4	...	91.8	94.9	...	...	25.2	58.1	19.6	79.6	72.8	42.0	...	...	7.2
7.4	...	...	...	...	...	...	19.7	57.6	...	...	...	9.8	30.6	55.8	...	93.0	95.8	...	...	26.7	55.3	14.6	71.3	72.8	42.0	...	...	7.4
7.6	...	...	...	...	...	...	12.8	60.8	...	...	...	6.8	29.6	58.2	...	93.8	96.8	...	...	28.6	...	10.2	62.0	63.2	39.3	...	...	7.6
7.8	...	...	...	...	...	...	7.4	65.2	53.0	...	...	4.6	28.8	60.5	...	...	...	...	...	31.2	...	6.6	52.0	52.1	33.7	43.9	...	7.8
8.0	...	...	...	...	...	...	3.7	70.6	55.4	...	...	...	28.1	62.8	...	...	...	...	...	33.9	...	...	42.0	41.1	27.9	41.6	...	8.0
8.2	...	...	...	...	...	...	...	75.9	58.0	...	...	...	27.6	65.0	...	...	...	...	46.4	36.9	...	...	31.9	31.4	22.9	38.4	...	8.2
8.4	...	...	...	...	...	...	...	81.2	62.1	...	...	...	27.0	67.2	...	...	...	...	43.9	39.9	45.4	...	22.5	23.0	17.3	34.8	...	8.4
8.6	...	...	...	...	...	...	...	86.2	66.9	94.7	...	...	26.3	69.3	...	...	...	45.5	40.9	42.7	42.8	...	16.0	15.9	13.0	30.7	...	8.6
8.8	...	...	...	...	...	...	...	90.1	73.6	92.0	...	...	25.2	71.3	...	...	...	45.5	36.8	...	42.8	...	11.7	10.3	8.8	23.3	...	8.8
9.0	...	...	...	...	...	...	...	93.2	83.5	88.4	...	...	24.0	73.2	...	...	...	43.2	31.8	...	39.2	...	...	...	5.3	17.7	...	9.0
9.2	...	...	...	...	...	...	...	...	95.6	84.0	...	...	22.6	75.1	...	...	...	40.0	26.2	...	34.7	...	...	...	...	13.3	10.0	9.2
9.4	...	...	...	...	...	...	...	...	...	78.9	87.0	...	21.4	77.0	...	...	...	35.8	20.4	...	29.3	...	...	...	...	9.2	18.4	9.4
9.6	...	...	...	...	...	...	...	...	...	73.2	75.5	...	20.2	78.8	...	...	...	30.8	15.2	...	23.6	...	...	...	...	5.2	29.3	9.6
9.8	...	...	...	...	...	...	...	...	...	67.2	65.1	...	19.0	80.4	...	...	...	25.0	10.8	...	19.0	...	...	...	...	4.1	42.0	9.8
10.0	...	...	...	...	...	...	...	...	...	62.5	59.6	...	18.1	81.8	...	...	...	19.4	7.4	...	13.1	...	...	...	...	2.3	53.4	10.0
10.2	...	...	...	...	...	...	...	...	...	58.8	56.4	...	17.1	83.1	...	...	...	14.3	...	...	9.2	...	...	...	...	...	63.7	10.2
10.4	...	...	...	...	...	...	...	...	...	55.7	54.1	...	16.5	84.3	...	...	...	10.0	...	...	6.2	...	...	...	...	...	73.1	10.4
10.6	...	...	...	...	...	...	...	...	...	53.6	52.3	...	16.0	85.4	...	...	...	6.9	...	...	...	...	...	...	...	...	81.2	10.6
10.8	...	...	...	...	...	...	...	...	...	52.2	...	...	15.5	86.5	...	...	...	...	...	...	...	...	...	...	...	...	87.9	10.8
11.0	...	...	...	...	...	...	...	...	...	51.2	...	...	14.7	87.8	...	...	...	...	...	...	...	...	...	...	...	...	...	11.0
11.2	...	...	...	...	...	...	...	...	...	50.4	...	...	13.5	89.3	...	...	...	...	...	...	...	...	...	...	...	...	...	11.2
11.4	...	...	...	...	...	...	...	...	...	49.5	...	...	11.7	91.3	...	...	...	...	...	...	...	...	...	...	...	...	...	11.4
11.6	...	...	...	...	...	...	...	...	...	48.7	...	...	9.1	94.5	...	...	...	...	...	...	...	...	...	...	...	...	...	11.6
11.8	...	...	...	...	...	...	...	...	...	47.6	...	...	5.5	99.0	...	...	...	...	...	...	...	...	...	...	...	...	...	11.8
12.0	...	...	...	...	...	...	...	...	...	46.0	...	...	1.3	...	...	...	...	...	...	...	...	...	...	...	...	...	...	12.0
12.2	...	...	...	...	...	...	...	...	...	43.2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	12.2
12.4	...	...	...	...	...	...	...	...	...	39.1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	12.4
12.6	...	...	...	...	...	...	...	...	...	31.8	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	12.6
12.8	...	...	...	...	...	...	...	...	...	21.4	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	12.8

EXPERIMENT IV

Isolation of Natural Compounds from Plant Sources

Natural compounds can be defined as organic molecules produced by living organisms. Approximately half of the pharmaceutical agents currently in use are known to be derived from natural sources. Plants and bacteria represent the most significant reservoirs for the discovery of new pharmacologically active compounds. Metabolic products that are essential for the survival of an organism are referred to as primary metabolites. An organism cannot survive if it fails to synthesize even one of its primary metabolites. In plants, amino acids, nucleic acids, pyruvate, and products of central metabolic pathways are examples of primary metabolites. In contrast, secondary metabolites are compounds that are not strictly necessary for basic survival but enhance an organism's fitness under specific environmental conditions. Antibiotics produced by bacteria and alkaloids and phenolic compounds synthesized by plants are typical examples of secondary metabolites.

Alkaloids are nitrogen-containing organic bases derived primarily from amino acid metabolism. Plants generally do not excrete nitrogen-containing compounds because assimilable nitrogen is often a limiting factor in plant growth. When sufficient nitrogen is available, certain amino acids may be synthesized in excess. These surplus amino acids can be converted into alkaloids, which accumulate as end products of specialized metabolic pathways.

The biosynthesis of plant secondary metabolites follows defined metabolic routes. Common precursor molecules for alkaloid biosynthesis include ornithine, lysine, phenylalanine, tyrosine, and nicotinic acid (Figure 1). Nicotine [1-methyl-2-(3-pyridyl)pyrrolidine] is found predominantly in tobacco plants and in smaller quantities in tomatoes, eggplants, and green peppers. The nitrogen atoms present in both the pyridine and pyrrolidine rings confer basic properties to the molecule. Nicotine has been used commercially as an insecticide and in veterinary medicine as a parasiticide. However, high doses are toxic to humans. It is a colorless, oily liquid with a boiling point of 246 °C and is readily soluble in water and many organic solvents. In plant tissues, nicotine exists in association with organic acids, particularly citric and malic acids (2–8% in dried tobacco). It can be extracted using dilute alkaline solutions. Following basification, nicotine is transferred into an organic phase such as ether and subsequently purified. Chemical characterization of nicotine can be challenging due to its liquid nature and the often limited quantities obtained during isolation. To facilitate isolation and

identification, nicotine may be reacted with picric acid to form nicotine dipicrate salt, which can be isolated in solid form.

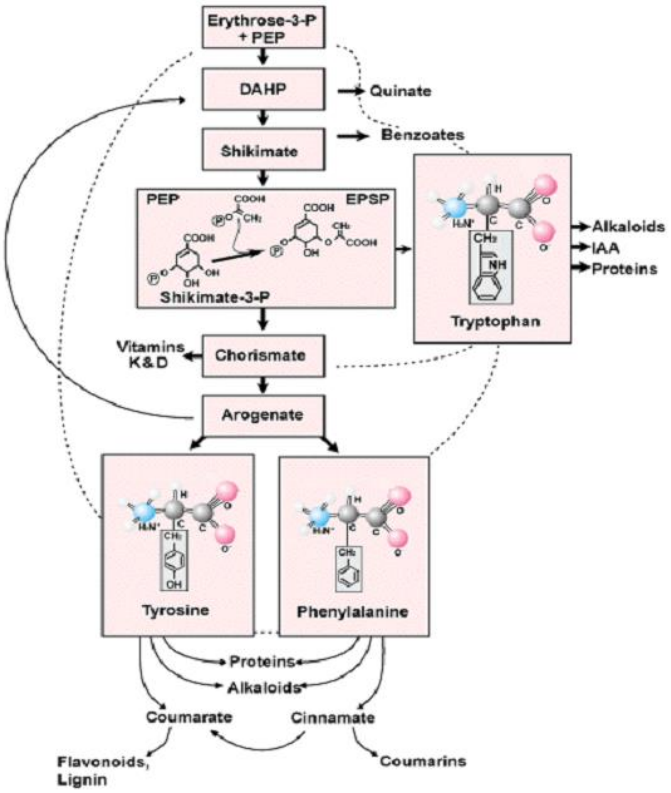


Figure 1. Overview of the shikimate pathway and its role in aromatic amino acid and secondary metabolite biosynthesis.

METHOD

Chemicals and Solutions Used in the Experiment

- 5% (w/v) NaOH solution (5 g NaOH / 100 mL water)
- Diethyl ether
- Saturated picric acid solution

Approximately 4 g of tobacco is weighed and placed into a 100 mL beaker. Then, 25 mL of 5% NaOH solution is added, and the mixture is crushed and stirred for 2–3 minutes using a glass rod to facilitate extraction. The resulting extract is filtered through a funnel containing glass wool and collected in a single Erlenmeyer flask. The alkaline extract is then transferred into a separatory funnel and extracted twice with 25 mL portions of diethyl ether. The combined diethyl ether extracts are evaporated to dryness under a fume hood. The residue is subsequently dissolved in 5 mL of water and 2–3 mL of ethanol. Approximately 5 mL of saturated picric acid

solution is then added. Yellow crystals of nicotine dipicrate are formed. The resulting crystals are filtered and dried. The melting point of the derivative is 222–224 °C.

The method described above is a specific procedure developed for the isolation of nicotine and cannot be universally applied to all natural compounds. It is often difficult to identify a selective precipitating agent for every natural product. Therefore, chromatographic techniques are commonly employed for the isolation of natural compounds. Adsorption chromatography, High-Performance Liquid Chromatography (HPLC), and Gas Chromatography (GC) are among the most widely used techniques for this purpose. It should also be noted that organisms do not continuously synthesize their secondary metabolites. These compounds are generally produced during specific developmental stages or under particular environmental conditions.

General Steps for the Isolation of Natural Compounds

The general procedure for isolating a compound from natural sources can be summarized as follows:

- First, determine the developmental stage and the specific organ or tissue (e.g., root, leaf, flower) in which the compound is synthesized in the highest concentration.
- Collect the relevant plant material during the identified period. The material is typically dried and may undergo preliminary fractionation processes.
- Select an appropriate solvent for extraction based on the chemical structure of the target compound. Solubility testing and literature review are essential at this stage.
- Homogenize the sample with the selected solvent and remove insoluble materials by centrifugation or filtration.
- Extract the solution with a suitable volatile organic solvent in which the compound is soluble. The choice of solvent is determined based on structural considerations and literature data.
- After extraction, evaporate the solvent and re-dissolve the residue in a small volume of solvent (e.g., methanol, ethanol, or water) to concentrate the sample.
- Separate the desired compound from the mixture using chromatographic techniques such as column adsorption chromatography, preparative thin-layer chromatography (TLC), or HPLC.
- Finally, confirm the identity of the isolated compound using analytical techniques such as mass spectrometry (MS) and/or nuclear magnetic resonance (NMR) spectroscopy.

QUESTION

1- Investigate how an organic compound is isolated from a natural source. Write a assay for this research area.

EXPERIMENT V

Protein Ligand Interactions: Binding of Coumarin Compound to Bovine Serum Albumin

Coumarins are secondary plant metabolites, and they have been found to be present in natural structures such as fungi, bacteria and stems, fruits, and flowers of vascular plants. Coumarins are divided into four main classes: simple coumarins (Figure 1), furanocoumarins, pyranocoumarins, and coumarins with substitutions in the pyrone ring. Coumarins have been discovered to have various pharmacological properties such as anti-coagulant, anti-neurodegenerative, anti-bacterial, antioxidant, anti-diabetic, anti-depression and anti-cancer.

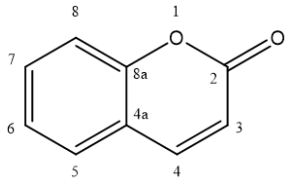


Figure 1. General structure of coumarin

Many therapeutic drugs do not achieve the desired effectiveness due to limitations in stability, half-life, and solubility. It is recommended to use drug carrier systems to protect substances from degradation, ensure efficacy, and avoid immune system rejection. As drugs are absorbed into the body and transported to target tissues via blood, interaction with blood proteins such as serum albumin (SA) is inevitable. Serum albumin (SA), one of the most common proteins found in the blood, has a primary function in the transport of endogenous and exogenous substances (fatty acids, drugs, metabolites, etc.), in addition to its different physiological functions such as adjusting osmotic pressure, determining blood pH, and reducing free radicals.

Understanding how a drug behaves in the body and its effects on disease conditions by determining its pharmacokinetic and pharmacological activity is crucial to providing effective treatment for patients. For these reasons, investigation of the binding properties of small molecules to optimal biomacromolecules is necessary, and it has an important field in biochemistry.

METHOD

When the 4-hydroxycoumarin compound binds to protein, the compound undergoes a spectral change that is quite apparent in the visible area. The spectrum of the free compound is around 340 nm. When the compound and protein are mixed, a new absorption band appears. The

increase in absorption at 340 nm is directly related to the interaction with protein. The new absorption band belongs to the coumarin - protein complex. At 280 nm, the bovine serum albumin (BSA) band, change occurs after interaction with the coumarin compound.

Chemicals and Solutions

Solution A: Phosphate buffer 0.01 M pH: 7.4.

Solution B: 5 ml methanol + 45 ml A solution.

Solution C: 0.001 g 4-hydroxycoumarin + 50 ml B solution.

Solution D: 0.1 g BSA 20 ml A solution.

- $Mw_{coumarin}$: 162.14 g/mol, Mw_{BSA}: 66430.3 g/mol

PROCESS

Working Solutions	Solution A (µL)	Solution C (µL)	Solution D (µL)
1	2500	-	500
2	2300	200	500
3	2100	400	500
4	1900	600	500
5	1700	800	500
6	1500	1000	500
7	1300	1200	500
8	1100	1400	500
9	900	1600	500
10	700	1800	500
11	500	2000	500
12	2400	600	-

Before measure spectrum the spectrophotometer (double beam) was reset with solution A. All spectra of the solutions to be measured will be between 200-600 nm.

DATA USE

"**A_0/(A-A_0)** (y-axis)" versus "**1/c** (x-axis)" is drawn (Don't forget to examine the R^2 value of the plot. It gives information about the accuracy of the graph). The slope of this plot gives binding constant (K_b).

- **"A_0"** is the first result of the spectrum (**1**) and is always the same value. "**A"** is the result of the spectra **2**, **3**, **4**, **5**, **6**, **7**, **8**, **9**, **10** and **11** and is variable. The 12th spectrum belongs to coumarin.
- **"c"** is the concentration of coumarin. This value is variable.

ΔG^o was calculated using the Gibbs-Helmholtz equation (below).

$$\Delta G^o = -RT\ln K_b$$

R= Universal gas constant, 8.314 $JK^{-1}mol^{-1}$, T= 298 K

QUESTIONS

1- Explain the three-dimensional structure of BSA and mention its binding sites.

2- What do ΔG and K_b indicate? What do they indicate thermodynamically? Explain the interaction mechanism of the coumarin compound with BSA by $\Delta G°$ and K_b values.

EXPERIMENT VI

Separation of Plant Pigments by Column Chromatography

Column chromatography is a general term used to describe chromatographic techniques performed in a column format. Ion-exchange chromatography, size-exclusion chromatography (molecular sieve chromatography), partition chromatography, adsorption chromatography, and affinity chromatography can all be conducted using column systems.

In adsorption chromatography, the column packing material (stationary phase) interacts with the components of a sample mixture with different affinities. The compound that interacts most strongly with the stationary phase will elute last from the column. In partition chromatography, the stationary phase typically exhibits similar interaction strength toward all components of the sample. In this case, molecules that interact more favorably with the mobile phase elute more rapidly from the column.

In column chromatography, the stationary phase is packed into a stopcock-equipped column and pre-equilibrated with the mobile phase. The sample is then applied to the top of the column. As the mobile phase slowly passes through the stationary phase, it carries the sample components along. The eluates are collected as separate fractions, and the composition of each fraction is determined using appropriate analytical techniques. A variety of materials may serve as stationary phases, including aluminum oxide, magnesium hydroxide, magnesium carbonate, magnesium silicate, zinc carbonate, calcium oxalate, calcium carbonate, calcium oxide, talc, silica gel, finely powdered glass, activated carbon, and even ordinary chalk. The stationary phase must be insoluble in the chosen eluent. Possible solvents include water, acids, bases, salt solutions, gasoline, petroleum ether, acetone, alcohols, carbon disulfide, cyclohexane, methyl ethyl ketone, and pyridine. Using this technique, it is possible to isolate multiple components from green leaf extracts. Key considerations in column chromatography include:

- Proper selection of the stationary phase
- Uniform packing of the column (avoiding air bubbles and channel formation)
- Appropriate solvent selection
- Controlled flow rate of the mobile phase
- Adequate separation of bands
- Efficient recovery of separated bands

METHOD

Approximately 3–4 g of fresh green leaves are cut into small pieces and homogenized in a mortar with approximately 16 mL of petroleum ether:diethyl ether:methanol (9:6:1, v/v/v). The resulting liquid extract is transferred into an evaporating dish and evaporated to dryness at low temperature.

Approximately 10 g of silica gel is moistened with petroleum ether and packed into a column using a funnel, ensuring that the material does not adhere to the column walls. The column stopcock remains closed during packing. The stopcock is then opened, and petroleum ether is carefully added from the top using a Pasteur pipette to wash down the column walls. This procedure is repeated until a clean and uniform surface is obtained. When the petroleum ether level reaches approximately 0.1 cm above the stationary phase surface, the stopcock is closed.

The dried extract residue is dissolved in the minimum possible volume of petroleum ether and carefully applied to the top of the column using a Pasteur pipette, avoiding disturbance of the stationary phase surface. The column is opened to allow dropwise elution, permitting the green solution to enter the column.

Shortly thereafter, a yellow carotene band becomes visible. When the carotene band has migrated approximately 1–2 cm, petroleum ether containing 10% chloroform is used as the mobile phase. The blue-green chlorophyll a band begins to migrate. By gradually increasing the chloroform concentration, first the yellow xanthophyll band and subsequently the yellow-green chlorophyll bands are eluted.

The carotene fraction collected from the bottom of the column is analyzed spectrophotometrically within the wavelength range of 200–900 nm.

For paper chromatography, a drop of the sample is applied approximately 1.5 cm above the bottom edge of a chromatography paper using a Pasteur pipette. After drying, the paper is developed in a chromatography chamber containing petroleum ether as the mobile phase. The resulting colored bands are recorded.

QUESTION

1- What are the phenolic compounds found in plants? What physiological effects do they exert? Investigate how a phenolic compound can be isolated from a plant source using HPLC and briefly describe the procedure.

EXPERIMENT VII

Extraction and Saponification of Milk Fats

The principal components of milk are carbohydrates (milk sugar, lactose, approximately 4.9%), lipids (approximately 3.7%, primarily in the form of butterfat), and proteins (casein, approximately 3%, and albumin, approximately 0.5%). In addition to these major constituents, milk contains small quantities of vitamins and inorganic salts, which, although present at low concentrations, are nutritionally significant.

The fatty acid composition of milk fat is as follows to Figure 1:

Fatty Acid Composition of Milk Fat

Fatty Acid	Percentage (%)
C16:0	30.51
C18:1 9-*cis*	19.73
C18:0	10.93
C14:0	10.53
C18:1	4.12
C4:0	2.65
C18:2	2.04
C16:1	1.39
C15:0	1.17
C18:3	0.96
Saturated	67.47
Monounsaturated	26.99
Polyunsaturated	4.24

Figure 1. Fatty acid composition (%) of milk fat.

Milk fats can be easily extracted using organic solvents. The general formula for these fats is as follows to Figure 2.

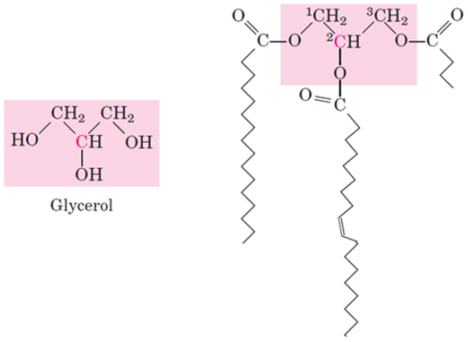


Figure 2. Structural representation of glycerol and a triglyceride (1-palmitoyl-2-oleoyl-3-butanoyl glycerol).

In the structure presented above, a representative triacylglycerol composed of the most abundant fatty acids in milk—namely palmitic acid and oleic acid—together with butyric acid, which is responsible for the characteristic aroma of butter, is illustrated as an example. However, this is not the only triacylglycerol present in milk fat. Milk fat contains a complex mixture of both homotriacylglycerols (triacylglycerols containing three identical fatty acid residues) and various heterotriacylglycerols (triacylglycerols containing different fatty acid residues). This compositional diversity contributes significantly to the physicochemical properties and sensory characteristics of milk fat. The saponification reaction of milk fats can be represented as follows to Figure 3:

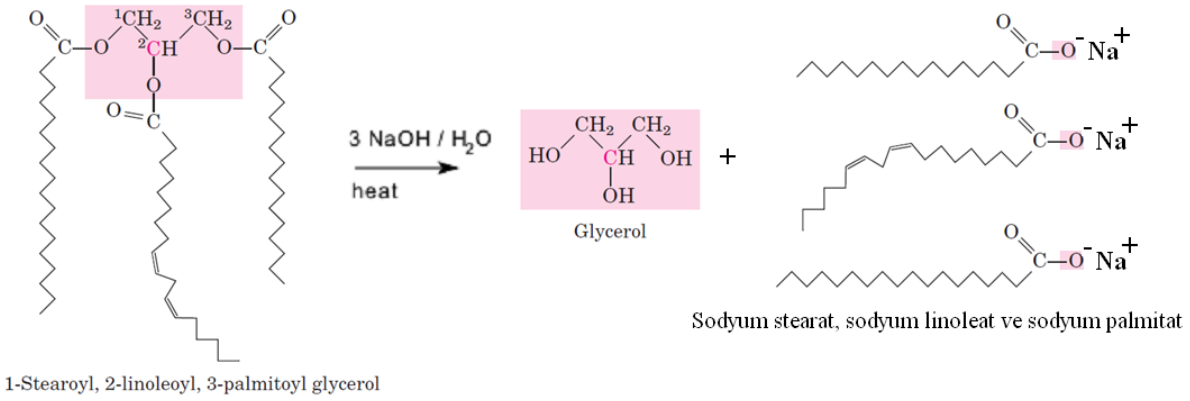


Figure 3. Saponification reaction of a triacylglycerol yielding glycerol and sodium salts of fatty acids.

The objective of this experiment is to perform the extraction of milk lipids, to determine the total fat content quantitatively, and to obtain the constituent fatty acids from these lipids through appropriate chemical processes.

METHOD

Chemicals

- 25% (w/v) ammonia solution (NH_3)
- Ethanol
- Diethyl ether
- Petroleum ether
- 10% (w/v) sodium hydroxide (NaOH)
- Concentrated hydrochloric acid (HCl)

Extraction of Milk Lipids

Milk lipids were extracted according to the Roese–Gottlieb method, a widely used procedure for the determination and isolation of milk fat.

To 1 mL of milk sample, 0.2 mL of 25% ammonia solution was added. The mixture was incubated in a water bath at 70 °C for 5 minutes with occasional mixing. Subsequently, the tube was cooled under running tap water.

Then, 1 mL of ethanol was added, and the tube was gently inverted for 1 minute without vigorous shaking to ensure proper mixing. Next, 2.5 mL of diethyl ether was added and mixed by gentle inversion for 1 minute. Afterward, 2.5 mL of petroleum ether was added and again mixed gently for 1 minute.

The mixture was allowed to stand on a flat surface for 5–10 minutes to permit phase separation. The clear ether layers were carefully collected into a beaker and evaporated to dryness on a hot plate.

Saponification of Milk Lipids

Approximately 0.5 g of the extracted fat was weighed and refluxed for approximately 45 minutes with 40 mL of methanol containing 10% NaOH.

After completion of reflux, the alcohol was evaporated. The residue was dissolved in a small amount of hot water and acidified with concentrated HCl. The liberated fatty acids were observed, and their physical appearance was recorded.

QUESTIONS

1- Define probiotics and prebiotics. What are their applications and significance?

2- What is the composition of milk, and what are its nutritional and physiological benefits? Investigate and discuss.

EXPERIMENT VIII

Simultaneous Determination of Vitamins E and C

The term vitamin E refers to a group of eight structurally related antioxidant compounds. Four of these compounds belong to the tocopherol family (α-, β-, γ-, and δ-tocopherols), while the remaining four are classified as tocotrienols (α-, β-, γ-, and δ-tocotrienols) (Figure 1).

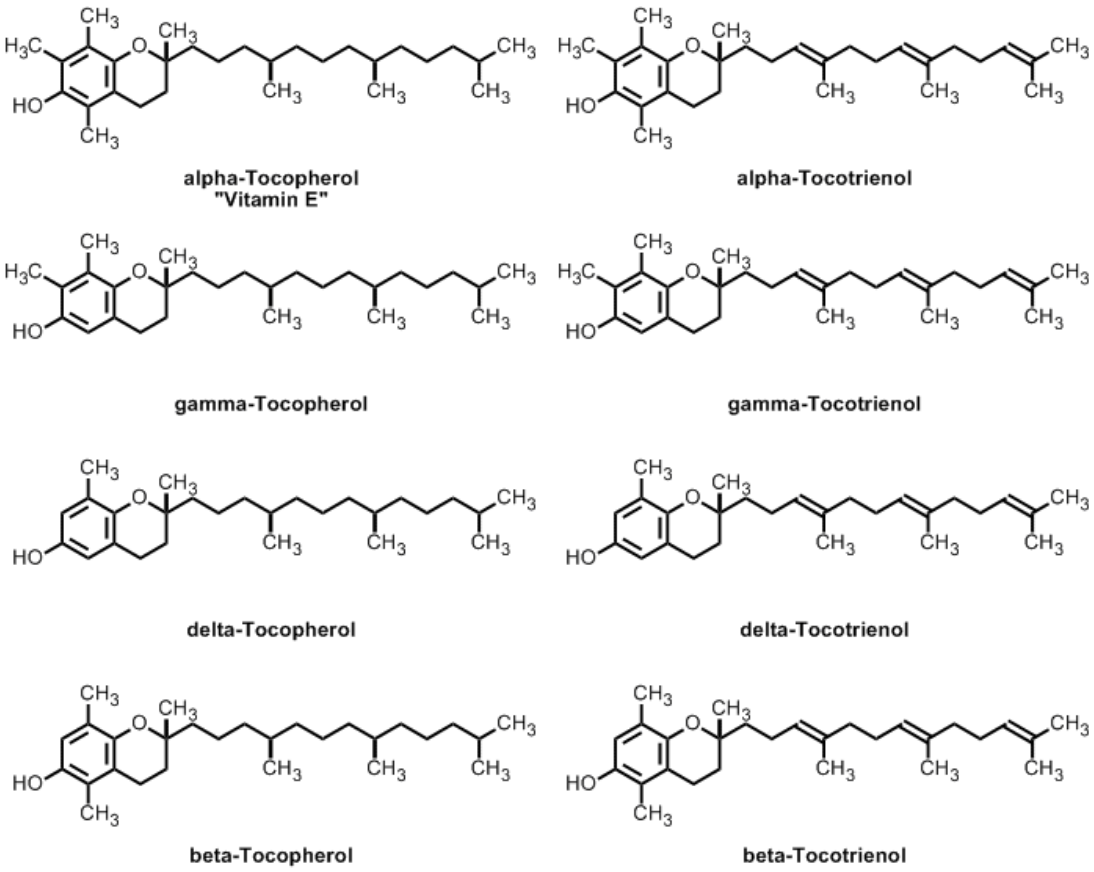


Figure 1. Chemical structures of tocopherols and tocotrienols (vitamin e isoforms).

The only form of vitamin E that is actively maintained at a constant level in the human body is alpha-tocopherol. In biological systems, it primarily functions as an antioxidant. During normal physiological processes, particularly aerobic respiration, various reactive oxygen species (ROS) and other free radicals are generated. These free radicals can attack macromolecules such as DNA, proteins, and lipids, leading to significant cellular damage. Alpha-tocopherol, being a fat-soluble vitamin, acts predominantly within lipid environments to prevent lipid peroxidation and oxidative degradation of fatty acids. When a molecule of alpha-tocopherol neutralizes a free radical, it temporarily loses its antioxidant capacity. However, other antioxidants, such as vitamin C (ascorbic acid), can regenerate alpha-tocopherol, restoring its antioxidant activity.

Both ascorbic acid and alpha-tocopherol exhibit antioxidant properties. The combined antioxidant effect of these two compounds is greater than the sum of their individual effects, demonstrating a synergistic interaction. For this reason, they are frequently used together in food preservation to enhance oxidative stability. Ascorbic acid is a water-soluble vitamin, whereas alpha-tocopherol is lipid-soluble. Nevertheless, both compounds are sufficiently soluble in absolute ethanol to allow detection in the ultraviolet (UV) region. Taking advantage of this property, the concentrations of vitamins E and C can be simultaneously determined in the UV spectral region.

METHOD

Chemicals and Solutions Used

- Ascorbic acid
- Alpha-tocopherol
- Ethanol

Preparation of Standard Solutions

Ascorbic acid (0.0050 g/L in absolute ethanol): Approximately 0.010 g of ascorbic acid is weighed into an Eppendorf tube and dissolved in 1 mL of ethanol. From this solution, 5 µL is taken and diluted to 10 mL with ethanol.

Alpha-tocopherol (0.0500 g/L in absolute ethanol): Approximately 0.002–0.003 g of alpha-tocopherol is weighed into an Eppendorf tube and dissolved in 1 mL of ethanol. The volume corresponding to 0.0005 g of the substance is calculated, transferred into a 10 mL volumetric flask, and diluted to volume with ethanol.

Preparation of Calibration Standards

From the ascorbic acid stock solution, aliquots of 0.2, 0.3, 0.4, and 0.5 mL are taken and each is diluted to 2.5 mL with absolute ethanol. Standard solutions of alpha-tocopherol are prepared in the same manner.

Absolute ethanol is used as the blank solution. The absorption spectra of the standard solutions are recorded between 220–320 nm. All four ascorbic acid concentrations are plotted on the same graph. Similarly, the spectrum of alpha-tocopherol is recorded. Finally, the spectrum of the unknown sample is obtained.

Calibration and Quantitative Analysis

From the standard curves of ascorbic acid and alpha-tocopherol, the wavelengths corresponding to maximum absorbance (λmax) for each compound are determined. Calibration curves are constructed by plotting absorbance values at the selected wavelength (y-axis) versus concentration (x-axis).

The molar absorptivity (extinction coefficient, ε) of each compound is calculated at the relevant wavelengths. Using these values, the concentrations of ascorbic acid and alpha-tocopherol in the unknown sample are determined.

QUESTIONS

1- What factors must be considered when determining two compounds simultaneously in a mixture?

2- What are Reactive Oxygen Species (ROS)? By which mechanisms are they generated in biological systems, and through which mechanisms are they neutralized?

EXPERIMENT IX and X

Purification and Activity Analysis of Amylase from Yeast

The amylase enzyme is a metalloenzyme found in high amounts in microorganisms, plants, animals, fungi, and especially in germinating grains. Based on its mechanism of action, amylase is classified as alpha (α), beta (β), and gamma (γ) (Figure 1). α-Amylase (EC 3.2.1.1) is produced in both the salivary glands and the secretory granules of pancreatic cells in mammals. This enzyme is also known as α-1,4-glucan-4-glucanohydrolase. Its activity depends on calcium and chloride ions. Additionally, it exhibits its highest activity at pH 7.

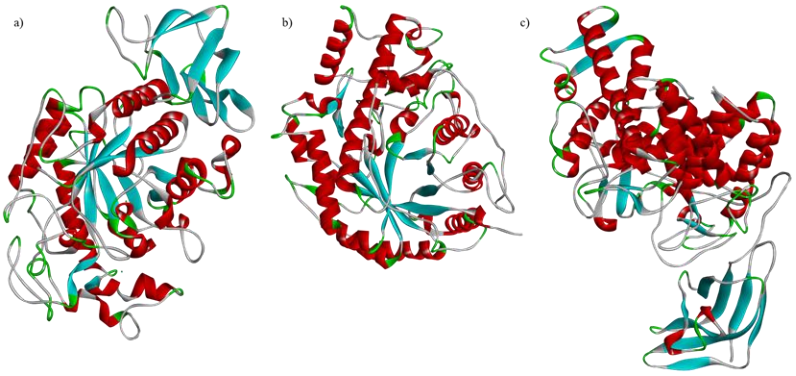


Figure 1. a) Structure of human α-amylase (PDB: 1SMD) b) Structure of barley β-amylase (PDB: 2XFR) c) Structure of Penicillium oxalicum γ-amylase (6FHV).

In order for carbohydrate molecules to be absorbed in the body, they must first be broken down into monosaccharide units by the α-amylase enzyme. Carbohydrates that begin to be digested in the mouth are not digested in the stomach, where α-amylase is inactive due to acidic environmental conditions. With the neutralization of pH in the small intestine, α-amylase is secreted from the pancreas and carbohydrate digestion continues there.

Purification of proteins (Figure 2) helps researchers understand the structure and function of a specific protein. Protein purification is a crucial process in biotechnology, biochemistry, and medicine. It is also a critical step in the production of biotechnological products such as enzymes, antibodies, and recombinant proteins. Purified proteins are essential for structural and functional analyses, drug production, and industrial applications. For example, therapeutic proteins like insulin are used to treat diseases, and these proteins must be obtained with high purity.

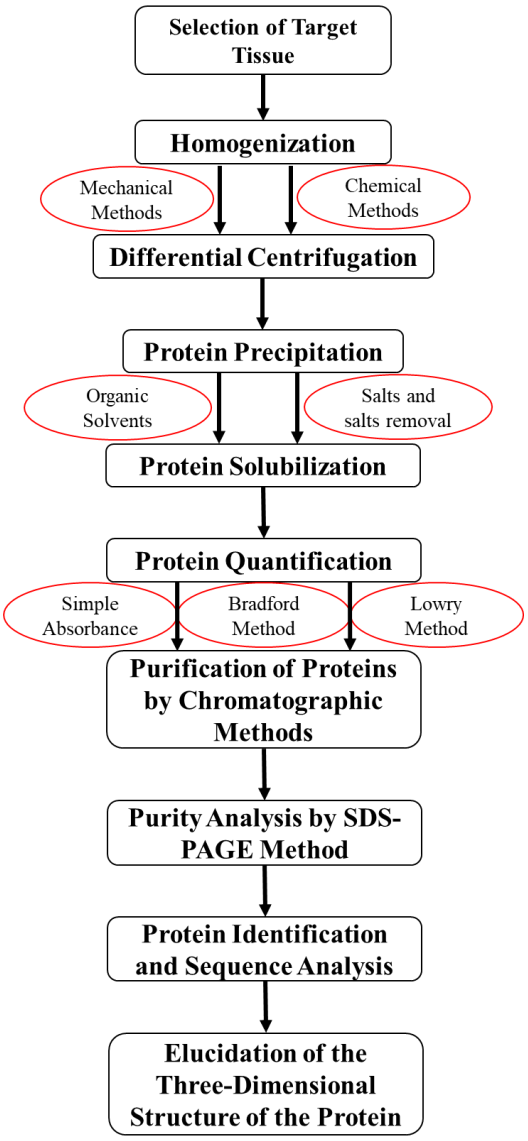


Figure 2. General purification process for protein.

1-) Selection of Target Tissue: The tissue must be suitable for isolating protein. It must be easily accessible, abundant, have low complex content and high protein content.

2-) Homogenization: Homogenization is a process used to break down biological materials to release cellular components. It is especially important for extracting intracellular proteins, enzymes, and other biomolecules. By disrupting cell membranes and organelles, homogenization helps prepare samples for purification and biochemical analysis. Homogenization methods are broadly categorized into mechanical methods and chemical methods.

- Mechanical methods: Freeze-Thaw Method, Ultrasonication (Sonication), Bead Beating (Bead Milling), Potter-Elvehjem Homogenizer (Tissue Homogenizer), and French Press apparatuses disrupt cell structures.

- Chemical methods: Detergents, enzymes, or hypotonic solutions to disrupt cell structures. Lysis buffer contain a buffering agent (e.g. Tris-HCl) to maintain the pH of the solution. It also typically contains salts (e.g. NaCl, KCl), detergents (e.g. SDS, Tween-20), nuclease inhibitors (e.g. EDTA), reducing agents (DTT), and protease inhibitors (e.g. PMSF).

3-) Differential Centrifugate: The technique relies on the fact that different cellular components sediment at different rates when subjected to centrifugal force. By applying sequential centrifugation steps at increasing speeds, cellular fractions such as nuclei, mitochondria, lysosomes, and cytoplasmic proteins can be separated efficiently.

- Low-Speed Centrifugation (800–1,000 × g, 5–10 min): This step removes large debris, unbroken cells, and nuclei. The pellet contains intact cells and nuclei, while the supernatant contains smaller organelles and soluble proteins.
- Medium-Speed Centrifugation (10,000–20,000 × g, 10–20 min): This step removes mitochondria, lysosomes, and peroxisomes. The pellet contains these organelles, and the supernatant carries smaller vesicles and cytoplasmic proteins.
- High-Speed Centrifugation (100,000 × g, 1 hour): This step removes microsomes, membrane fragments, and ribosomes. The pellet contains membrane-bound organelles, and the supernatant holds soluble cytoplasmic proteins.
- Ultracentrifugation (Over 100,000 × g, 1–3 hours): This step is used to purify ribosomes and smaller macromolecules. The final supernatant contains purified soluble proteins.

4-) Protein Precipitation: Protein precipitation is a fundamental technique in protein purification used to concentrate and partially purify proteins by reducing their solubility in solution. This process is widely applied in biochemistry and biotechnology to isolate proteins from biological mixtures. Protein precipitation methods generally fall into two main categories: organic solvents and salts.

- Organic solvents: Ethanol, methanol, and acetone, are used to precipitate proteins by reducing the dielectric constant of the solution. This causes proteins to aggregate and precipitate out of the solution. Organic solvents decrease water's ability to solvate proteins, leading to protein aggregation. Solvents also disrupt hydrogen bonds and hydrophobic interactions, further reducing solubility. In this technique all kinds of proteins in the solution are precipitated. If the precipitation process is carried out in a

cold temperature, the proteins will precipitate without being denatured, whereas if it is carried out in a room (or higher) temperature, the proteins will precipitate as denatured.

- Salts: Salt precipitation, commonly known as salting out, relies on the principle that high salt concentrations compete with proteins for water molecules, reducing protein solubility and causing precipitation. At low salt concentrations, proteins remain soluble due to the stabilizing effect of salt ions (salting in). At high salt concentrations, proteins aggregate and precipitate as water molecules become less available for solvation (salting out). After salt precipitation, additional processing is required to remove salt from the solution.
    - *Dialysis*: Dialysis is a widely used technique in protein purification. In this process, the protein solution is placed inside a semi-permeable membrane (dialysis tubing), and this membrane allows small molecules like salts to pass through, but it retains the larger protein molecules.
    - *Gel Filtration (Size Exclusion Chromatography)*: Gel filtration, or size exclusion chromatography, is a technique used to separate molecules based on their size. In this method, a gel matrix (typically made of agarose or polymer-based materials) serves as a filtration medium to separate proteins from smaller molecules such as salts.

5-) Protein Solubilization: After precipitation of proteins with organic solvent, proteins need to be solubilized. For this purpose, proteins are solubilized using minimum volumes of buffers.

6-) Protein Quantification: Protein quantification refers to the process of determining the concentration of a protein in a given sample. There are various methods for protein quantification, such as Simple absorbance, Bradford and Lowry methods.

- Simple absorbance method: Tyrosine and tryptophan residues of the proteins have a maximum absorbance at 280 nm.
- Bradford method: Coomassie Brilliant Blue G250 (CBB) dye reacts with basic amino acid residues in the protein and is measured at a wavelength of 595 nm.
- Lowry method: Biuret reaction (peptide bonds react with alkaline copper solutions to form Cu+) coupled with Folin-Ciocalteau reaction (Phosphomolybdotungstic acid under Cu+ catalysis react with aromatic amino acids to form heteropolymolibden blue) are coupled and the absorbance is measured at a wavelength of 750 nm.

7-) Purification of Proteins by Chromatographic Methods: One of the most powerful and widely used techniques for protein purification is chromatography. Chromatography relies on the differential interaction of proteins with a stationary phase (solid or liquid) and a mobile phase (liquid or gas) to separate proteins based on various properties such as size, charge, hydrophobicity, and affinity for specific ligands.

- Ion Exchange Chromatography (IEX): Ion exchange chromatography is based on the charge properties of proteins. It separates proteins based on their net charge at a given pH.
- Gel Filtration Chromatography (Size Exclusion Chromatography, SEC): Gel filtration chromatography separates proteins based on their size (molecular weight). It is useful for both protein purification and for determining the size and oligomeric state of proteins.
- Affinity Chromatography: Affinity chromatography is based on the specific binding between a protein and a ligand, such as an antibody, enzyme substrate, or metal ion.
- Thin Layer Chromatography (TLC): While not commonly used for large-scale protein purification, TLC can be employed to monitor protein purification and to separate small amounts of proteins.

After purification by chromatography, protein quantification is performed again.

8-) Purity Analysis by SDS-PAGE Method: SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) is one of the most commonly used techniques for determining the purity of proteins, as well as for analyzing their molecular weight. SDS-PAGE works by separating proteins based on their size and charge after denaturation with SDS, a detergent that imparts a negative charge to the proteins. This method provides information about protein purity, molecular weight, and the presence of any impurities or degradation products.

- Sample Preparation: Protein sample is mixed with SDS loading buffer containing SDS, reducing agents (such as DTT or β-mercaptoethanol), and a tracking dye. The sample is heated (typically at 95-100 °C) for 5-10 minutes to ensure complete denaturation.
- Gel Casting: The polyacrylamide gel is prepared, usually consisting of a stacking gel (with a lower concentration of acrylamide) and a separating or resolving gel (with a higher concentration of acrylamide).

- Electrophoresis: The protein samples are loaded into the wells of the gel, and an electric field is applied. Proteins migrate through the gel matrix, with the rate of movement inversely proportional to their size.
- Staining and Visualization: After electrophoresis, the gel is stained with Coomassie Brilliant Blue (which binds to proteins) or silver staining for more sensitive detection of low-abundance proteins. The gel is then visualized, and the bands are compared with the molecular weight marker to determine the sizes of the proteins.

9-) Protein Identification and Sequence Analysis: Protein identification and sequence analysis are critical steps in proteomics and molecular biology, allowing researchers to determine the identity and structure of proteins. Various methods are employed to identify proteins and analyze their sequences, including mass spectrometry (MS), Edman degradation, and bioinformatics tools.

- Mass spectrometry (MS): It allows for high-throughput identification of proteins in complex biological samples by measuring the mass-to-charge ratio (m/z) of ions.
    - Proteins are typically digested into smaller peptides (using proteases like trypsin) and ionized. These peptides are then analyzed in a mass spectrometer, where they are separated based on their mass-to-charge ratio. The resulting data are used to identify the peptides and, ultimately, the proteins from which they originated.
- Western Blotting: Western blotting is a widely used technique to identify specific proteins based on their antigenic properties. It involves detecting proteins through specific antibodies.
    - After separating proteins by SDS-PAGE, proteins are transferred to a membrane (e.g., nitrocellulose or PVDF), and then probed with antibodies specific to the target protein. The antibody is conjugated with a detectable label (e.g., enzyme, fluorescence).
- Protein Microarrays: Protein microarrays are a high-throughput technique used to analyze protein-protein interactions, binding affinity, and protein expression.
    - Protein microarrays contain thousands of distinct proteins immobilized on a solid surface. The protein of interest is exposed to various probes (e.g., antibodies, peptides, or other proteins) to identify binding partners.

- Edman Degradation (N-terminal Sequencing): Edman degradation is a classical method for determining the amino acid sequence of proteins, particularly their N-terminal sequence.
    - The protein is cleaved one residue at a time from the N-terminus by reacting it with phenylisothiocyanate (PITC). The resulting cyclic derivative is identified, and the process is repeated for each successive amino acid.

10-) Elucidation of the Three-Dimensional Structure of the Protein: The three-dimensional (3D) structure of a protein is closely related to its function, and understanding this structure can provide insights into its biological role, mechanisms of action, and potential for drug development or therapeutic applications. Several techniques are employed to determine the 3D structure of proteins, including X-ray crystallography, Nuclear Magnetic Resonance (NMR) spectroscopy, Cryo-Electron Microscopy (Cryo-EM), and computational methods. Below is an overview of these methods and their applications.

- X-ray Crystallography: X-ray crystallography has been the most widely used method for determining the 3D structure of proteins at atomic resolution. It involves analyzing the diffraction pattern of X-rays passed through a protein crystal.
    - The protein must first be crystallized into a highly ordered structure. X-rays are then directed at the crystal, and the diffraction pattern produced by the crystal is recorded. From the diffraction data, electron density maps are generated, which are then used to model the protein's 3D structure.
- Nuclear Magnetic Resonance (NMR) Spectroscopy: NMR spectroscopy is another technique for determining the 3D structure of proteins, particularly useful for studying proteins in solution.
    - NMR spectroscopy detects the magnetic properties of atomic nuclei (such as hydrogen, carbon, or nitrogen) within a protein. By analyzing the interactions between these nuclei, NMR provides distance restraints that can be used to determine the 3D structure of the protein in solution.
- Cryo-Electron Microscopy (Cryo-EM): Cryo-EM is a powerful technique for determining the 3D structures of large, flexible macromolecular complexes and is becoming increasingly popular for protein structure determination.
    - In cryo-EM, a protein sample is rapidly frozen to preserve it in a near-native state and then imaged using an electron microscope. Thousands of 2D images

are captured from different orientations, and computational techniques are used to reconstruct the 3D structure.

METHOD

Purification of amylase from yeast (*Saccharomyces cerevisiae*) and determination of enzyme activity will be performed.

Chemicals and Solutions

Acetone, ammonium sulfate, Tris-HCl, serum physiologic solution, BSA, Bradford reagent, 3,5-dinitrosalicylic acid (DNS), $CaCl_2$.

PROCESS

Amylase enzyme will be isolated from instant yeast using two different precipitation methods: organic solvent and salt precipitation.

1. Organic solvent: Protein precipitation with acetone is typically is performed at a 1:9 ratio, meaning 1 part sample will mix with 9 parts acetone. This method reduces protein solubility, leading to precipitation. After precipitation, centrifugation is performed to obtain the protein precipitate. After centrifugation, the protein will dissolve with the minimum amount of buffer and protein quantification will perform. If the protein ratio gives a positive result, enzyme activity will be determined.
2. Salts: Protein will separate into four fractions with ammonium sulfate (0–30%, 30–60%, 60–90%, and 90–100%). The precipitated proteins will collect by centrifugation at 10,000×g for 10 min at 4 °C, and the pellet from each fraction will dissolved in 5 mL of 50 mM Tris-HCl buffer (pH 7.0). Then, the resuspended protein precipitates will dialysis overnight against 0.25 L of the same buffer (three changes) at 4 °C with continuous mixing to completely remove ammonium sulfate.

Protein quantification assay for both precipitation methods: Simple Absorbance (Table 1) and Bradford methods (Table 2) will be used for protein quantification.

Table 1. Process of Simple Absorbance Method.

Tube No	BSA Stok (mL)	Serum physiologic (mL)	Absorbance (280 nm)	Protein Concentration (µg/mL)
1	0	3.0		
2	0.5	2.5		
3	1.0	2.0		
4	1.5	1.5		
5	2.0	1.0		
Sample	0.1	2.9		

Serum physiologic solution: 0.897% NaCl (250 mL).

BSA stock solution: 100 mL 0.2 BSA% solution (prepared in Serum physiologic solution).

Prepare protein standard solutions according the following table and measure the absorbance of standard solutions and samples at 280 nm. Draw the calibration plot (concentration (µg/mL) vs. absorbance) using the standard protein solutions and from the equation of the plot calculate the concentration of the samples.

Table 2. Process of Bradford Method.

Tube No	Protein Stok (µL)	Water (µL)	Bradford Reagent (µL)	Mix and incubate for 10 min.	Absorb. (595 nm)	Protein Concentration (µg/mL)
1	0	800	200			
2	20	780	200			
3	40	760	200			
4	60	740	200			
5	80	720	200			
Sample	2	798	200			

Protein Stock solution: Ovalbumin stock solution (2 mg/mL) should be diluted 20 times to a final concentration of 0.1 mg/mL. Bradford reagent should be at room temperature. Prepare protein standard solutions according the following table and measure the absorbance of standard solutions and samples at 595 nm. Draw the calibration plot (concentration (µg/mL) vs. absorbance) using the standard protein solutions and from the equation of the plot calculate the concentration of the samples.

Enzyme activity assay for both precipitation methods: Amylase enzyme activity determination will be studied using the 3,5-dinitrosalicylic acid (DNS) method. 0.5 mL of dialyzed, partially purified enzyme will be added to 0.5 mL of 1% (w/v) soluble starch in 50 mM Tris-HCl buffer (pH 7.0) containing 5 mM $CaCl_2$. The test tubes will be covered and incubated for 5 minutes at

65 °C in a water bath. Then, 1 mL of DNS reagent will be added to each tube to stop the reaction, followed by placement in a boiling water bath for 5 minutes. After cooling the samples in a cold water bath, the absorbance will be read at 540 nm. All eluted fractions will be assessed for enzyme activity, and absorbance will be measured at 540 nm and 575 nm, respectively. The fractions showing the highest enzyme activity will be pooled and analyzed for protein content. The specific activity of purified enzyme fractions will be compared to that of the crude enzyme.

QUESTIONS

1- Inhibition of amylase enzyme is used in the treatment of which disease? Give information about this disease.