SYLLABUS

1. Partial purification of acid phosphatase from germinating mung bean.

2. Assay of enzyme activity and specific activity, e.g. acid phosphatase.

3. Effect of pH on enzyme activity

4. Determination of Km and Vmax using Lineweaver-Burk graph.

5. Enzyme inhibition - calculation of Ki for competitive inhibition.

6. Continuous assay of lactate dehydrogenase.

7. Assay of glucose-6-phosphate dehydrogenase.

KAHE

1. PARTIAL PURIFICATION OF ACID PHOSPHATISE FROM GERMINATED MUNG BEAN

1.A. AMMONIUM SULPHATE PRECIPITATION

AIM

To precipitate the protein content by ammonium sulphate precipitation method using various concentrations of ammonium sulphate.

THEORY

Precipitation is the method of concentrating proteins prior to analysis for a subsequent purification step. Protein precipitate is formed by the aggregation of protein molecules induced by changing of pH or ionic strength or by addition of organic solvents or other inert solutes or polymers. The solubility of a protein molecule in an aqueous solvent is determined by the distribution of changed hydrophyllic and hydrophobic groups.

PRINCIPLE

Ammonium sulphate is particularly a useful salt in the fractional precipitation of proteins which precipitates the protein by changing its ionic strength of the medium as the ionic strength increase protein solubility.

This is referred to as ‘salting in’ however at a certain point the solubility starts to decrease, and this is known as ‘salting out’.

Salting out is depends on the hydrophobic nature of the surface of the proteins, hydrophobic groups predominate in the interior of the protein but some are located at the surface often in patches. When the protein is in solution, water molecules are surrounds the protein forming hydrogen bonds with the protein. When salts are added to the system, water solubilises the ions and as the salt concentration increases, water is removed around the protein eventually exposed in the hydrophobic patches. Hydrophobic patches on one protein molecule can interact with those on another resulting in aggregation. Thus protein with large or more hydrophobic patches will aggregate and precipitate the before with smaller or fewer hydrophobic patches resulting in fractionation, hydrophobic proteins precipitate out first. But the hydrophyllic proteins precipitate with much higher salt concentration. Advantages of ammonium sulphate are high solubility, low cost and easy availability. It is also non-toxic to most enzymes and it has stabilizing effect to all enzymes.

MATERIALS REQUIRED

1. Reagents

   Extract preparation

   Mung bean seeds were collected from local market and 10g of seeds are weighed and washed using distilled water and soaked with 20ml of distilled water in petridish and incubated at 20◦C for 24 hours to induce germination. After 24 hours the seeds were crushed using mortar and pestle with appropriate solutions and made upto 100 ml with the same buffer solution and filtered. This filtrate is used as crude extract.
2. Ammonium sulphate
3. Citrate buffer

   Solution A:

   Dissolve 21.01g of citric acid in 1 litre of distilled water.

   Solution B:

   Dissolved 21.41g of sodium citrate in 1 litre of distilled water.

PROCEDURE

1. The prepared crude extract is transferred to the beaker placed in a tray.
2. Check the ammonium sulphate chart and weigh the appropriate amount of ammonium sulphate to get 30% solution fraction of protein.
3. Slowly add ammonium sulphate crystal with crude extract collected in beaker and dissolved it completely. Vigorous stirring will lead to denaturation of protein.
4. Keep the saturated solution undisturbed at 4◦C for 30-60 minutes.
5. Then transferred the solution to centrifuge tubes and spin at 1000rpm for 20 minutes at 4◦C.

6. Carefully transferred a supernatant to beaker placed in an ice tray.
7. Dissolved the pellet in 1ml of buffer.
8. Put the supernatant again and slowly add ammonium sulphate up to the fdinal concentration of 60% mixing continuously until its dissolve completely
9. Repeat the steps 4,5,6 and 7
10. Again slowly add ammonium sulphate to the supernatant to a final concentrations of 90% repeat the steps 4,5,6 and 7
11. All the three pellets dissolved in minimal volume of extraction buffer and dialysed against the same buffer with constant stirring for overnight
12. It was used for acid phosphatise activity assay and the protein concentration is measured throughout the purification process by the method of process given by lowry's method 1951 using BSA as standard protein.

1.b. ESTIMATION OF PROTEIN

Lowry *et al.*, 1957

AIM

To estimate the amount of protein present in the given sample.

PRINCIPLE

The blue color developed by the reduction of the phosphomolybdic phosphotungstic components in the Folin-Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the color developed by the biuret reaction of the protein with the alkaline cupric tartarate are measured at 660nm.

Reagents

1. 2% Sodium carbonate in 0.1N NaOH (Reagent A)
2. 0.5% Copper sulphate in 1% potassium sodium tartarate (Reagent B)
3. Alkaline copper reagent:

Mixed 50ml of A and 0.1ml of B prior to use.

4. Folin-Ciocalteau reagent:

Mixed 1 part of reagent with 2 parts of water.

5. Stock standard:

Weighed 50mg of bovine serum albumin and made up to 50ml in a standard flask with saline.

6. Working standard:

Diluted 10ml of the stock with 50ml of distilled water. 1.0ml of this solution contains 200µg of protein.

PROCEDURE

Pipetted out 0.2 to 1.0ml working standard solution, 0.1ml of the sample was taken. The volume in all the tubes was made up to 1.0ml with distilled water. Added 5.0ml of alkaline copper reagent to each tube. Mixed well and allowed to stand for 15 minutes. Then added 0.5ml of Folin-Ciocalteau reagent. Mixed well and incubated at room temperature for 30 minutes. A reagent blank was also prepared. After 30 minutes, the blue color developed was read at 660nm. A standard graph was drawn by plotting the concentration on x axis and the optical density on y axis. From this, the concentration of protein in the given sample solution was calculated.

RESULT

The amount of protein present in the given solution was found to be ---------- g/dl

2. ASSAY OF ENZYME ACTIVITY AND SPECIFIC ACTIVITY OF ACID PHOSPHATASE

King, 1965

AIM

To estimate the amount of acid phosphatase present in the given sample.

PRINCIPLE

The method used was that of King and Armstrong in which disodium phenylphosphate is hydrolyzed with the liberation of phenol and inorganic phosphate. The liberated phenol is measured at 700nm with Folin-Ciocalteau reagent.

REAGENTS

1. Citrate buffer: 0.1M, pH 5.

   A: Citric acid (21.01g in 100ml)

   B: Sodium citrate (29.41g in 100ml)

2. Disodium phenyl phosphate, 100mmol/L:

   Dissolved 2.18g of disodium phenylphosphate in distilled water and heated to boil, cooled and made to a litre. Added 1.0ml of chloroform and stored in the refrigerator.

3. Buffered substrate:

   Prepared by mixing equal volume of the above two solutions. This has a pH of 5.0.

4. Folin-Ciocalteau reagent:

   Prepared by mixing one volume of reagent and two volumes of water. Sodium carbonate solution, 15%: Dissolved 15g of anhydrous sodium carbonate in 100ml water.

5. Standard phenol solution, 1g/L:

   Dissolved 1g pure crystalline phenol in 100mmol/L HCl and made to a litre with acid.

6. Working standard solution:

   Diluted 10ml of stock standard to 100ml with water. This contains 100µg of phenol/ml.

PROCEDURE

Pipetted out 4.0ml of buffered substrate into a test tube and incubated at 37˚C for 5 minutes. Added 0.2ml of sample and incubated further for exact 60 minutes. Removed and immediately added 1.8ml of diluted phenol reagent. At the same time, set up a control containing 4.0ml buffered substrate and 0.2ml of sample to which 1.8ml of phenol reagent was added immediately. Mixed and centrifuged. To 4.0ml of supernatant added 2.0ml of 15% sodium carbonate. Took 4.0 ml of working standard solution and for blank taken 3.2 ml water and 0.8ml of phenol reagent. Then added 2.0 ml of sodium carbonate. Incubated all the tubes at 37˚C for 5 minutes. Read the color developed at 700nm.

A standard graph was drawn by plotting the concentration on x axis and the optical density on y axis. From this, the concentration of acid phosphatase in the given sample solution was calculated.

RESULT

The activity of acid phosphatase was found to be ------------- µmoles of phenol liberated per litre.

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3. EFFECT OF PH ON ENZYME ACTIVITY

AIM:

To find the effect of pH on acid phosphatase activity.

Principle:

This enzyme catalyses the hydrolysis of phosphate ester to phosphoric acid and alcohol. The amount of phosphoric acid produced during hydrolysis is the measure of enzyme activity. The liberated phosphoric acid containing the inorganic phosphorous is estimated by fiske and subbarow method.

Reagents

- Citrate buffer (0.2M)
- Sodium β glycero phosphate (substrate) (0.1M)
- 15% Trichloro acetic acid
- Ammonium molybdate solution
- ANSA reagent
- Magnesium acetate (0.2M)

PREPARATION OF BUFFER

pH	Solution A: x ml of 0.2 M citric acid (ml)	Solution B: y ml of 0.2 M sodium citrate	H_2O
4.6	12.75	12.25	25
5.0	10.25	14.75	25
5.6	6.85	18.15	25
6.0	4.75	20.25	25
6.6	2.5	22.5	25

Procedure:

Citrate buffer of various pH (4.6-6.4) is pipetted out into a clean dry test tubes.Then added 0.5ml of megnesium acetate solution and 1ml of the substrate to each followed by the addition of 2.0ml of the enzyme. Incubated at 37ºC for 1hour. At the end of incubation period added 1.0ml of 15% trichloroacetic acid to stop the reaction. Controls were also conducted along with these to which the enzyme was added at the end of incbation period.

Standard phosphate solution in the range 1µg to 8 µg were pipetted out into clean dry test tubes .This serves as the standard to produce the standard graph.

All the test tubes are centrifged and the supernatant of 1ml is transfered to other tubes, Added 1ml of molybdate solution followed by 0.4ml of ANSA and made upto 10ml with distilled water.The colour developed was read at 660nm after 20 minutes.The enzyme activity at various pH is found out from the standard graph.

Result:

The activity of the enzyme acid phosphatase was found to be maximum at pH ___ for an incubation period of 1 hour at 37ºC.

EFFECT OF PH ON ACID PHOSPHATASE ACTIVITY

Ph		**4.6**	**5.0**	**5.6**	**6.0**	**6.6**
Volume of citrate buffer (ml)		**5.0**	**5.0**	**5.0**	**5.0**	**5.0**
Volume of magnesium acetate (ml)		**0.5**	**0.5**	**0.5**	**0.5**	**0.5**
Volume of substrate (ml)		**1.0**	**1.0**	**1.0**	**1.0**	**1.0**
Volume of enzyme (ml)		**2.0**	**2.0**	**2.0**	**2.0**	**2.0**
Incubate at 37ºC for one hour						
Volume of 10% TCA		**1.0**	**1.0**	**1.0**	**1.0**	**1.0**
Add enzyme to the control and centrifuge at 5000 rpm for 10 minutes						
Volume of supernatant (ml)		**1.0**	**1.0**	**1.0**	**1.0**	**1.0**
Volume of ammonium molybdate (ml)		**1.0**	**1.0**	**1.0**	**1.0**	**1.0**
Volume of ANSA (ml)		**0.4**	**0.4**	**0.4**	**0.4**	**0.4**
Volume of water (ml)		**7.6**	**7.6**	**7.6**	**7.6**	**7.6**
Colorimeter reading	**Control**					
	Experiment					
	Difference					

4. DETERMINATION OF KM AND VMAX USING LINEWEAVER-BURK GRAPH.

Aim:

To find the effect of substrate concentration on acid phosphatase activity.

Principle:

This enzyme catalyses the hydrolysis of phosphate ester to phosphoric acid and alcohol. The amount of phosphoric acid produced during hydrolysis is the measure of enzyme activity. The liberated phosphoric acid containing the inorganic phosphorous is estimated by Fiske and Subbarow method.

Procedure:

Into clean dry test tubes pipetted out 0.5ml of magnesium acetate solution and varying volume of substrates (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0ml) and made up to 7.0ml with 0.2M citrate buffer of pH 5.6 (test) followed by the addition of 1.0 ml of the enzyme. Incubated at 37 ºC temperature for one hour, stop the reaction by the addition of 2.0ml of 10%TCA. Along with this control is also conducted to which the enzyme was added at the end of the incubation period. Centrifuged and transferred 1ml of the supernatant of each tubes to other tubes. Added 1ml of the ammonium molybdate solution followed by 0.4 ml of ANSA reagent. Made up the volume of each tube to 10 ml with distilled water. Read the colour developed at 660nm in a colorimeter after 20 minutes. A Michaelis Menton plot graph was drawn by plotting the substrate variation on x axis and colorimeter reading on y axis. From this the Vmax, 1/2Vmax and km values were calculated.

A Line Weaver burk plot was also prepared by plotting the reciprocal of the substrate concentration on x axis and the reciprocal of the optical density on y axis. The graph was plotted to get the value of Km.

Result:

The value of Km from Michaelis Menton plot=1/2 Vmax=

The value of Km from Line Weaver burk plot =1/2 Vmax=

EFFECT OF SUBSTRATE CONCENTRATION ON ACID PHOSPHATASE ACTIVITY

S.NO		1	2	3	4	5	6	7
Volume of substrate (ml)		0.5	1.0	1.5	2.0	2.5	3.0	3.5
Volume of citrate buffer (ml)		6.5	6.0	5.5	5.0	4.5	4.0	3.5
Volume of enzyme (ml)		0.5	0.5	0.5	0.5	0.5	0.5	0.5
Volume of magnesium acetate (ml)		0.5	0.5	0.5	0.5	0.5	0.5	0.5
Incubate at 37ºC for one hour								
Volume of 10% TCA		2.0	2.0	2.0	2.0	2.0	2.0	2.0
Add enzyme to the control and centrifuge at 5000 rpm for 10 minutes								
Volume of supernatant (ml)		1.0	1.0	1.0	1.0	1.0	1.0	1.0
Volume of ammonium molybdate (ml)		1.0	1.0	1.0	1.0	1.0	1.0	1.0
Volume of ANSA (ml)		0.4	0.4	0.4	0.4	0.4	0.4	0.4
Volume of water (ml)		7.6	7.6	7.6	7.6	7.6	7.6	7.6
Colorimeter reading	Control							
	Experiment							
	Difference							

Calculation

Molecular weight of sodium β – glycerol phosphate = 315.4 g

31.54 g of sodium β – glycerol phosphate was dissolved in a litre of distilled water

The molarity of sodium β – glycerol phosphate = $\frac{31.54}{315.4}$ **= 0.1M**

Dilution factor and substrate concentration in each tube

$$\frac{molarity}{dilution\ factMor} = \text{substrate concentration}$$

0.5 ml of substrate was diluted to 10 ml = $\frac{10}{0.5}$ = 20, $\frac{0.1}{20}$ = 0.5 x 10^{-2} M

1.0 ml of substrate was diluted to 10 ml = $\frac{10}{1.0}$ = 10, $\frac{0.1}{10}$ = 1.0 x 10^{-2} M

1.5 ml of substrate was diluted to 10 ml = $\frac{10}{1.5}$ = 6.66, $\frac{0.1}{6.66}$ = 1.5 x 10^{-2} M

2.0 ml of substrate was diluted to 10 ml = $\frac{10}{2.0}$ = 5, $\frac{0.1}{5}$ = 2.0 x 10^{-2} M

2.5 ml of substrate was diluted to 10 ml = $\frac{10}{2.5}$ = 4, $\frac{0.1}{4}$ = 2.5 x 10^{-2} M

3.0 ml of substrate was diluted to 10 ml = $\frac{10}{3.0}$ = 3.33, $\frac{0.1}{3.33}$ = 3.0 x 10^{-2} M

3.5 ml of substrate was diluted to 10 ml = $\frac{10}{3.5}$ = 2.85, $\frac{0.1}{2.85}$ = 3.5 x 10^{-2} M

Line Weaver Burk plot

[S] X 10^{-2}m	V	1/[S] X 10^{-2}m	1/[V]
0.5		2.0	
1.0		1.0	
1.5		0.6	
2.0		0.5	
2.5		0.4	
3.0		0.3	
3.5		0.2	

5. ENZYME INHIBITION - CALCULATION OF Ki FOR COMPETITIVE INHIBITION

AIM

To study the effect of metal ions in inhibiting enzyme activity.

PROCEDURE

The mixture, consisting of 100 µl of the 0.1M cation solution under test, 600 µl of 1M acetate buffer pH 5.5 and 100 µl of enzyme solution, was pre-incubated for 10 min at 37°C. After preincubation, 200 µl of 20 mM p-nitrophenyl phosphate was added to determine the activity as usual. Simultaneously control and blank experiments were run in which the cation and enzyme solutions were replaced by water, respectively, in the pre-incubation mixtures. Similarly, the effect of some compounds reacting with SH-groups of the enzyme on the enzyme activity at pH values varying from 3 to 9 was determined as described above. Kinetic parameters: The Km, Vmax and Ki values were determined using p-nitrophenyl phosphate as the substrate in concentrations of 0.06 - 4 mM in the absence or presence of two or three fixed concentrations of inhibitors. These kinetic parameters were determined from Line-weaver-Burk plots. Straight lines were drawn by applying least square rule. The pH dependence studies of Km ,Vmax and specificity constants were carried out.

6. CONTINUOUS ASSAY OF LACTATE DEHYDROGENASE.

AIM

To assay the activity of lactate dehydrogenase in the given sample.

PRINCIPLE

The lactate is acted upon by lactate dehydrogenase to form pyruvate in the presence of NAD. The pyruvate forms pyruvate phenyl hydrazone with 2,4 dinitrophenyl hydrazine. The color developed is read in a spectrophotometer at 440nm.

REAGENTS

1. Glycine buffer, 0.1M, pH 10: 7.505 g of glycine and 5.85 g of sodium chloride were dissolved in 1 litre of water.
2. Buffered substrate: 125 ml of glycine buffer and 75 ml of 0.1 N NaOH were added to 4 g of lithium lactate and mixed well.
3. Nicotainamide Adenine Dinucleotide: 10 mg of NAD was dissolved in 2.0 ml of water.
4. 2, 4 Dinitrophenyl hydrazine: 20 mg of DNPH was dissolved in 100 ml of 1 N HCl.
5. 0.4 N NaOH.
6. Standard, 1µmol/ml: 11 mg of sodium pyruvate was dissolved in 100 ml of buffered substrate (1 µmole of pyruvate/ml).
7. NADH solution, 1 µmol/ml: 8.5 mg/10 ml buffered substrate.

PROCEDURE

Placed 1.0 ml of buffered substrate and 0.1 ml of sample into each of two tubes. Added 0.2 ml of water to the blank. Then to the test added 0.2 ml of NAD. Mixed and incubated at

37°C for 15 min. Exactly after 15 min, 1.0 ml of dinitrophenyl hydrazine was added to each (test and control). Left for further 15 min. Then added 10 ml of 0.4N sodium hydroxide and the color developed was read immediately at 440 nm. A standard curve with sodium pyruvate solution with the concentration range 0.02 - 0.10µmole was taken.

LDH activity in serum was expressed as µmoles of pyruvate liberated/L and in tissue homogenate as nmoles of pyruvate liberated/min/mg protein.

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7. ASSAY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

AIM

To assay the activity of glucose-6-phosphate dehydrogenase in the given sample.

PRINCIPLE

Glucose-6-phosphate dehydrogenase is assayed by measuring the increase in absorbance, which occurs at 340 nm when NADP reduces to glucose-6-phosphate to NADP in the reaction catalyzed by glucose-6- phosphate dehydrogenase.

REAGENTS

- 0.1M Tris-HCl buffer, pH 8.2
  - A: 0.1M solutions of Tris (12.1g/1000 ml water)
  - B: 0.1 M HCl
- Mixed 50 ml of solution A and 21.9 ml of B and diluted to a total of 200 ml
- 0.2 mM NADP
- 0.1M magnesium chloride
- 6 mM glucose-6-phosphate

PROCEDURE

0.4 ml of Tris-HCl buffer, 0.2 ml of NADP, 0.2 ml of magnesium chloride, 1.0 ml water and 0.2 ml of enzyme were taken in a cuvette. The reaction was started by the addition of 0.2 ml of glucose-6-phosphate and the increase in OD was measured at 340 nm. The activity was expressed in terms of units/mg protein, in which one unit is equal to the amount of enzyme that brought about a change in OD of 0.01/min.

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Coimbatore – 641 021.

LECTURE PLAN
DEPARTMENT OF BIOCHEMISTRY

STAFF NAME: Dr.K.POORNIMA
SUBJECT NAME: ENZYMES PRACTICAL
SUB.CODE:17BCU212
SEMESTER: II CLASS: I B.Sc (BC)

Sl. No	Duration of Period	Topics to be Covered	Support material
		ENZYMES - PRACTICALS	
1	3	Partial purification of acid phosphatase from germinating mung bean.	J1:16777-16782
2	3	Assay of enzyme activity and specific activity, e.g. acid phosphatase.	J3:743-756
3	3	Effect of pH on enzyme activity	
4	3	Determination of K_m and V_{max} using Lineweaver-Burk graph.	
5	3	Enzyme inhibition - calculation of K_i for competitive inhibition.	
6	3	Continuous assay of lactate dehydrogenase	T1331-334:
7	3	Assay of glucose 6 phosphate dehydrogenase	J2: 313-315
8	3	Model practical examinations	

REFERENCES

1. T1: Dr.S.Rajan (2012) manual for medical laboratory technology-first edition : anjanaa book. house, Chennai.
2. J1: Surchandra *et al*.,(2012). African journal of biotechnology: 11(103) 16777-16782
3. J2: Balinsky.D and bernstern.R.E. 1963: Biochemistry, biophysics aeta 67:313-315.
4. J3: A.K.M. Asaduxaman *et al* ,(2011) Arch.bio.sci Bdqrade 63(3).

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Semester II

17BCU212 ENZYMES PRACTICAL 3H-2C

1. Partial purification of acid phosphatase from germinating mung bean.
2. Assay of enzyme activity and specific activity, e.g. acid phosphatase.
3. Effect of pH on enzyme activity
4. Determination of Km and Vmax using Lineweaver-Burk graph.
5. Enzyme inhibition - calculation of Ki for competitive inhibition.
6. Continuous assay of lactate dehydrogenase.
7. Assay of glucose-6-phosphate dehydrogenase.

REFERENCE

Nelson, D.L. and Cox, M.M., (2013). Lehninger: Principles of Biochemistry 6th ed.,

W.H. Freeman and Company (New York), ISBN:13: 978-1-4641-0962-1 /

ISBN:10:1-4292-3414-8.

Donald, V., and Judith G.V., (2011). Biochemistry 4th ed., John Wiley & Sons Asia Pvt. Ltd. (New Jersey), ISBN:978-1180-25024.

Nicholas C.P. and Lewis S., (1999). Fundamentals of Enzymology 3rd ed., Oxford University Press Inc. (New York), ISBN:0 19 850229 X.

VIVA QUESTIONS

1. What are the factors influencing enzyme catalyzed reaction?
2. What is the effect of enzyme concentration?
3. What is the effect of substrate concentration?
4. What is km value and what is its importance?
5. What is the optimum temperature of enzymes present in human body?
6. Give optimum pH of acid phosphatase?
7. What is competitive inhibition and give examples?
8. What is allosteric inhibition? Give example of allosteric inhibitor and allosteric activator?
9. What is the principle of protein estimation?
10. What is the principle of acid phosphatase estimation?
11. How will you extract protein from a source?
12. What is meant by ammonium sulphate precipitation?
13. What is the principle of column chromatography?
14. How will you collect the fractions in column chromatography
15. What is the optimum temperature of acid phosphatase?
16. Give any two rich sources of acid phosphatase.
17. What is the principle behind LDH estimation?
18. How will you carry out protein estimation?
19. What is the end point of protein estimation by Lowry's method?
20. How are enzymes classified?
21. What is the nm range in which protein is estimated?
22. What is the source you used for protein extraction?
23. What is meant by specific activity?
24. Give the formulae to calculate specific activity
25. What is the use of assessing the Km value of an enzyme? What is the application?

26. What is the effect of temperature on enzyme activity?
27. What is the effect of pH on the activity of an enzyme?
28. What is the explanation for the effect of pH?
29. What is zymogen?
30. What is allosteric inhibition?
31. What is covalent modification?
32. What is the significance of ACP?
33. What is the significance of LDH?
34. What are the main theoretical models that try to explain the formation of the enzyme-substrate complex?
35. Does pH affect enzyme activity?
36. What are enzyme cofactors**?**

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