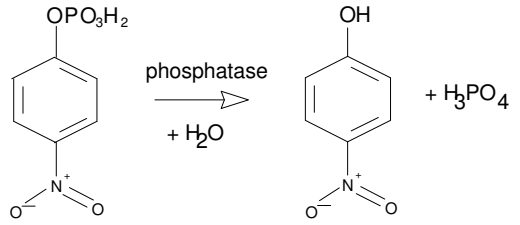
EXPERIMENT 3

STUDIES ON PHOSPHATASE

Phosphatases are widely distributed enzymes, they can be found in almost all living organisms. They split phosphate ester bonds liberating inorganic phosphate. Although most of the phosphatases have narrow substrate specificity, acid phosphatase - what is used in our experiments - is specific only for phosphomonoesters. It can split the phosphomonoester bond of various natural substrates (e.g. glycerol-phosphate, glucose-6-phosphate, fructose-1,6-diphosphate, adenosine monophosphate, etc.) and also different synthetic compounds. In our experiment p-nitrophenyl phosphate (pNPP, a synthetic substrate) is used.

The enzyme cleaves the ester bond of the colorless pNPP in acidic medium (pH=5.3) liberating inorganic phosphate and p-nitrophenol (pNP). The latter compound has a yellow color in alkaline medium. Absorption maximum of pNP is at 410 nm where its molar absorption coefficient is 1.6×10^7 $M^{-1}.cm^{-1}$.



p-nitrophenyl phosphate (pNPP, colorless) p-nitrophenol (pNP, yellow)

1. Preparation of p-nitrophenol reference curve

Prepare a reference curve for the determination of the amount of pNP.

Materials, Equipment

30 mM NaOH solution
0.1 mM p-nitrophenol in 30 mM NaOH solution
test tubes, pipettes
photometer set to 400 nm
cuvettes

Procedure

Prepare the following reaction mixtures:

	1.	2.	3.	4.	5.	6.	7.
Standard pNP solution (ml)	0.2	0.5	1.0	1.5	2.0	2.5	3.0
NaOH solution (ml)	4.8	4.50	4.0	3.5	3.0	2.5	2.0

Total volume: 5.0 - 5.0 ml

Measure the absorbance of the samples at 400 nm. Adjust the photometer to zero using distilled water.

Write the A_{400} values into a table and plot the absorbance values against the pNP content of the samples. Check whether the Lambert-Beer law is valid under these circumstances. With the help of the reference curve determine the A_{400} value of 0.1 µmol p-nitrophenol and use it for subsequent calculations.

2. Determination of the time-dependence of the enzyme reaction

Materials, Equipment

25 mM Tris-citrate buffer, pH=5.3
10 mM p-nitrophenyl phosphate solution
0.4 mM p-nitrophenyl phosphate solution
acid phosphatase solution
0.1 M NaOH solution
test tubes, pipettes
water bath at 37 °C
stopwatch
photometer set to 400 nm
cuvettes

Prepare the following reaction mixtures:

	1.	2.	3.	4.	5.	6. Blank
10 mM pNPP solution (ml)	1.000	1.000	1.000	1.000	1.000	1.000
Tris-citrate buffer (ml)	1.00	1.000	1.000	1.000	1.000	1.000
Incubate the test tubes in a water bath for 5 minutes at 37 °C, then add						
Enzyme solution (ml)	1.000	1.000	1.000	1.000	1.000	-
Distilled water (ml)	-	-	-	-	-	1.000

Incubate the test tubes for 5, 10, 15, 20, 30 minutes, respectively, then stop the reaction by adding 2 ml of 0.1 M NaOH solution. Mix carefully and measure the absorbance of the samples at 400 nm. Adjust the photometer to zero using distilled water. Calculate the amount of liberated pNP and the reaction rate for each sample.

Repeat the experiment using 0.4 mM substrate solution!

Tabulate the result as follows:

number of test tube	reaction time (s)	A400	$A_{400} - A_{400,\ blank}$	pNP (µmol)	reaction rate (µM/s)

Plot the amounts of pNP and the reaction rates against the time of incubation. Plot your data measured at two different concentration of the substrate on the same graph-paper!

3. Substrate concentration dependence of the reaction rate

Materials, Equipment

25 mM Tris-citrate buffer, pH=5.3
25 mM Tris-citrate buffer containing 0.3 mM K_2HPO_4, pH=5.3
25 mM Tris-citrate buffer containing 0.3 mM NaF, pH=5.3
A substrate (0.375 mM pNPP)
B substrate (0.6 mM pNPP)
C substrate (1.0 mM pNPP)
D substrate (3.0 mM pNPP)
acid phosphatase solution
0.1 M NaOH solution
test tubes, pipettes

photometer set to 400 nm
cuvettes
water bath at 37 °C

Procedure

Prepare the following reaction mixtures:

	1.	2.	3.	4.	5.	6.	7.	8.
Tris-citrate buffer (ml)	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
A substrate solution (ml)	1.000	1.000	-	-	-	-	-	-
B substrate solution (ml)	-	-	1.000	1.000	-	-	-	-
C substrate solution (ml)	-	-	-	-	1.000	1.000	-	-
D substrate solution (ml)	-	-	-	-	-	-	1.000	1.000
Enzyme solution (ml)	1.000	-	1.000	-	1.000	-	1.000	-
Distilled water (ml)	-	1.000	-	1.000	-	1.000	-	1.000

Incubate the test tubes in water bath at 37 °C for 15 minutes. Add 2 ml of NaOH solution to each then measure the absorption at 400 nm. Read at first the blanks (tubes 2, 4, 6, 8), then the samples in order of the increasing intensity of color (1, 3, 5, 7). Adjust the photometer to zero using distilled water.

Calculate the amount of released pNP and the rate of the reaction. Tabulate the result as follows:

Number of test tube	S (µM)	A_{400}	$A_{400} - A_{400,\ blank}$	pNP (µM)	v (µM/s)

S is the substrate concentration in the reaction mixture, v is the reaction rate.

Construct a table as follows:

Number of test tube	S (µM)	1/S (μM^{-1})	v ($\mu M s^{-1}$)	1/v ($\mu M^{-1} s$)

Plot the reaction rate against substrate concentration and the reciprocal rate against reciprocal substrate concentration (Lineweaver-Burk method). Read the $1/K_M$ and the 1/vmax values from the graph and calculate the K_M and the Vmax values of the acid phosphatase - pNPP reaction.

4. Competitive inhibition with inorganic phosphate

Inorganic phosphate inhibits phosphatases competitively. Examine the effect of increasing substrate concentration on the reaction rate in the presence of inorganic phosphate solution. Determine the KM and the Vmax values found in the presence of 0.1 M potassium hydrogen phosphate.

Procedure

Prepare the following reaction mixtures:

	1.	2.	3.	4.	5.	6.	7.	8.
Tris-citrate buffer containing K_2HPO_4 (ml)	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
A substrate solution (ml)	1.000	1.000	-	-	-	-	-	-
B substrate solution (ml)	-	-	1.000	1.000	-	-	-	-

C substrate solution (ml)	-	-	-	-	1.000	1.000	-	-
D substrate solution (ml)	-	-	-	-	-	-	1.000	1.000
Enzyme solution (ml)	1.000	-	1.000	-	1.000	-	1.000	-
Distilled water (ml)	-	1.000	-	1.000	-	1.000	-	1.000

Incubate the test tubes in water bath at 37 °C for 15 minutes. Add 2 ml of NaOH solution to each then measure the absorption at 400 nm. Read at first the blanks (tubes 2, 4, 6, 8), then the samples in order of the increasing intensity of color (1, 3, 5, 7). Adjust the photometer to zero using distilled water.

Calculate the amount of released pNP and the rate of the reaction. Tabulate the result as follows:

Number of test tube	S (μM)	A_{400}	$A_{400} - A_{400,\ blank}$	pNP (μM)	V (μM/s)

Construct a table as follows:

Number of test tube	S (μM)	1/S (μM^{-1})	V (μMs^{-1})	1/v ($\mu M^{-1}s$)

Plot the reaction rate against substrate concentration and the reciprocal rate against reciprocal substrate concentration on the same graph-paper as you did it in case of part 3. Read the 1/KM and the 1/vmax values from the graph and calculate the KM and the Vmax values. Compare them to the control values (determined without any inhibitor).

5. Non-competitive inhibition with NaF

Sodium fluoride inhibits acid phosphatase since it reacts with the Zn^{2+} ion located in the active center of the enzyme. Examine the effect of increasing substrate concentration on the reaction rate in the presence of NaF. Determine the KM and the Vmax values found in the presence of 0.1 M NaF.

Procedure

Prepare the following reaction mixtures:

	1.	2.	3.	4.	5.	6.	7.	8.
Tris-citrate buffer containing NaF (ml)	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
A substrate solution (ml)	1.000	1.000	-	-	-	-	-	-
B substrate solution (ml)	-	-	1.000	1.000	-	-	-	-
C substrate solution (ml)	-	-	-	-	1.000	1.000	-	-
D substrate solution (ml)	-	-	-	-	-	-	1.000	1.000
Enzyme solution (ml)	1.000	-	1.000	-	1.000	-	1.000	-
Distilled water (ml)	-	1.000	-	1.000	-	1.000	-	1.000

Incubate the test tubes in water bath at 37 °C for 15 minutes. Add 2 ml of NaOH solution to each then measure the absorption at 400 nm. Read at first the blanks (tubes 2, 4, 6, 8), then the samples in order of the increasing intensity of color (1, 3, 5, 7). Adjust the photometer to zero using distilled water.

Calculate the amount of released pNP and the rate of the reaction. Tabulate the result as follows:

number of test tube	S (μM)	A_{400}	A_{400} - $A_{400,\ blank}$	pNP (μM)	V (μM/s)

Construct a table as follows:

number of test tube	S (μM)	1/S (μM^{-1})	V (μMs^{-1})	1/v ($\mu M^{-1}s$)

Plot the reaction rate against substrate concentration and the reciprocal rate against reciprocal substrate concentration on the same graph-paper as you did it in case of part 3 and 4. Read the 1/KM and the 1/vmax values from the graph and calculate the KM and the Vmax values. Compare them to the results obtained from the previous parts of the experiment.

Questions, Problems

1. What can we conclude from the reaction rate - reaction time function? How is it influenced by the enzyme - substrate ratio? What kind of problem can it cause in practice? (enzyme diagnostics!)
2. What are the KM and V_{max} and why is it important to know these values?
3. What are the advantages and disadvantages of the double reciprocal plot?
4. How do kinetic parameters change in case of competitive and non-competitive inhibition?
5. Why can you compensate the effect of competitive inhibitor by adding substrate in contrast with the non-competitive one?