FOSFATASA ÁCIDA DE GERMEN DE TRIGO: EFECTO DE LA INHIBICIÓN POR PRODUCTO SOBRE LOS PARÁMETROS CINÉTICOS DE UNA ENZIMA MULTISUSTRATO

¿QUÉ DEBES SABER?

- La diferencia entre un ensayo enzimático continuo y uno a tiempo fijo.
- Qué reacciones catalizan las fosfatasas y por qué se clasifican en ácidas o básicas.
- Qué controles se requieren para realizar mediciones de concentración de producto de manera espectrofotométrica y en qué rango de valores existe un rango confiable de absorbancia.
- Cuál es el principio del método de cuantificación espectrofotométrica de p-nitrofenol presente en soluciones básicas.

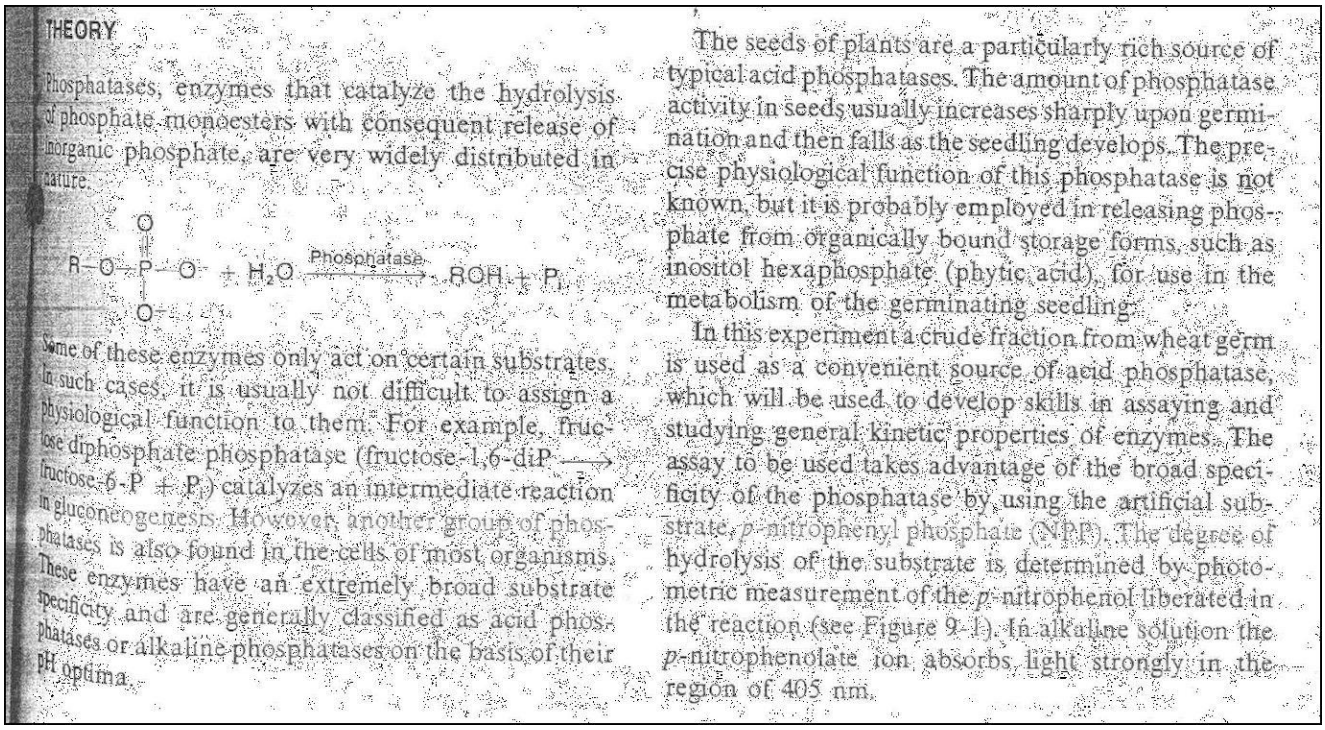
¿QUÉ REALIZARÁS?

Estudiarás la cinética enzimática de la enzima fosfatasa de germen de trigo utilizando como sustrato *p - nitrofenil fosfato (pNPP)*. La reacción catalizada produce *fosfato inorgánico (Pi) y p – nitrofenol (pNP)* siendo este último compuesto el responsable de entregar **en medio básico** una señal analítica útil para el estudio cinético de la enzima.

El ensayo enzimático es a tiempo fijo, por lo cual, a determinados tiempos extraerás alícuotas del medio de ensayo y detendrás la catálisis previo a medir el producto presente.

MATERIAL DE ESTUDIO

Con el siguiente texto podrán saber el fundamento del ensayo a realizar. El objetivo de este material es que sean capaces de diseñar y proponer su propio protocolo para estudiar cómo varían los parámetros cinéticos de la enzima ante inhibición por producto, para luego sugerir un posible mecanismo bisustrato de catálisis enzimática.



THEORY

Phosphatases, enzymes that catalyze the hydrolysis of phosphate monoesters with consequent release of inorganic phosphate, are very widely distributed in nature.

$$R-O-\overset{\overset{O}{\|}}{\underset{\underset{O^-}{|}}{P}}-O^- + H_2O \xrightarrow{\text{Phosphatase}} ROH + P_i$$

Some of these enzymes only act on certain substrates. In such cases, it is usually not difficult to assign a physiological function to them. For example, fructose diphosphate phosphatase (fructose-1,6-diP ⟶ fructose-6-P + P_i) catalyzes an intermediate reaction in gluconeogenesis. However, another group of phosphatases is also found in the cells of most organisms. These enzymes have an extremely broad substrate specificity and are generally classified as acid phosphatases or alkaline phosphatases on the basis of their pH optima.

The seeds of plants are a particularly rich source of typical acid phosphatases. The amount of phosphatase activity in seeds usually increases sharply upon germination and then falls as the seedling develops. The precise physiological function of this phosphatase is not known, but it is probably employed in releasing phosphate from organically bound storage forms, such as inositol hexaphosphate (phytic acid), for use in the metabolism of the germinating seedling.

In this experiment a crude fraction from wheat germ is used as a convenient source of acid phosphatase, which will be used to develop skills in assaying and studying general kinetic properties of enzymes. The assay to be used takes advantage of the broad specificity of the phosphatase by using the artificial substrate, *p*-nitrophenyl phosphate (NPP). The degree of hydrolysis of the substrate is determined by photometric measurement of the *p*-nitrophenol liberated in the reaction (see Figure 9-1). In alkaline solution the *p*-nitrophenolate ion absorbs light strongly in the region of 405 nm.

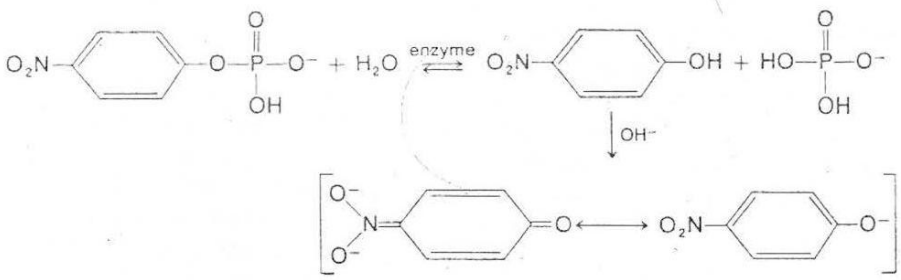


FIGURE 9-1
p-Nitrophenal phosphatase assay of phosphatase.

EXPERIMENTAL PROCEDURE

Materials

0.15 M Ethylenediamine–0.10 M citrate buffers of pH 3.0, 4.0, 4.5, 5.0, 5.5, 6.0, 7.0, and 8.0
0.025 M Disodium *p*-nitrophenyl phosphate (pH 4.8)
2.5 mM Disodium *p*-nitrophenyl phosphate (pH 4.8)
} NPP (Keep cold)
60 μM *p*-Nitrophenol in 0.02 M sodium hydroxide
0.02 M Sodium hydroxide
0.10 M Sodium hydroxide
Stock solution of crude wheat germ acid phosphatase (2 mg/ml in Triton X-100)
0.05 M Sodium phosphate (pH 4.8)
0.10 M Sodium fluoride
0.10 M Citrate buffer (pH 4.8)
0.1% Bovine serum albumin

Standard Curve for *p*-Nitrophenol Formation

If you are able to use a spectrophotometer and standard cells with a 1.0 cm light path, you can use the extinction coefficient for *p*-nitrophenol in 0.02 M NaOH ($E^{1M}_{1cm} = 18.8 \times 10^3$ at 405 nm) to calculate the μmoles of product formed from your absorbance readings. However, if you use a colorimeter and round colorimeter tubes, you must determine the relation between absorbance and μmoles of *p*-nitrophenol experimentally for your apparatus. Prepare 6 tubes containing 0.0, 1.0, 2.0, 3.0, 4.0 and 5.0 ml of the 60 μM *p*-nitrophenol solution and bring each tube to a total volume of 6.0 ml by addition of 0.02 M NaOH. Mix well, transfer a sample to a colorimeter tube and read the *A* at 405 nm. Use the sample containing no *p*-nitrophenol as a blank to adjust the colorimeter to zero absorbance. Plot A_{405} (ordinate) against the μmoles of *p*-nitrophenol in the sample. Use this standard curve for analysis of your kinetic data. Does this determination of *p*-nitrophenol obey the Lambert-Beer law?

Range Finding

Because the assay for phosphatase is a fixed time assay (see page 83), you must determine an appropriate dilution of stock enzyme solution that will yield a constant rate of product formation throughout the assay period. This is particularly important in this experiment because phosphatase is subject to product inhibition (see following discussion). Prepare a tube containing 2.0 ml ethylenediamine-citrate buffer, pH 5, and 2.0 ml 2.5 mM *p*-nitrophenyl phosphate (NPP). Place the tube in a 37° C water bath and allow a few minutes for it to come to temperature. Dilute the stock phosphatase solution to 10.0 ml with 0.1% bovine serum albumin. Add 1.0 ml of diluted enzyme to the tube and mix. *Immediately* transfer a 0.5 ml aliquot of the reaction mixture to a test tube containing exactly 5.5 ml of 0.1 M NaOH. This stops the enzyme-catalyzed reaction and makes the pH alkaline for measurement of *p*-nitrophenol. This sample is a "zero-time" control. Exactly 15 and 30 min after adding the enzyme to the tube, remove 0.5 ml samples and transfer them to tubes containing 5.5 ml of 0.1 M NaOH. Determine the absorbance at 405 nm of these tubes against the zero-time tube as a blank. Is the amount of *p*-nitrophenol formed (μmoles) linear with time over the 30 min period? (Note: In calculating the μmoles of *p*-nitrophenol formed in this and subsequent experiments, remember that the volume of assay tube was 5 ml and that an aliquot was diluted to 6 ml for absorbance measurements.) If it is not, test other dilutions of the stock phosphatase until a dilution is found that yields a linear response.

Determination of the pH Optimum

Prepare a series of 8 tubes. Each tube should contain 2.0 ml of an ethylenediamine-citrate buffer that has one of the following pH values: 3.0, 4.0, 4.5, 5.0, 5.5,

(U, 7.0 and 8.0. Add 2.0 ml of 2.5 mM NPP to each. Dilute the stock phosphatase solution with 0.1% bovine serum albumin to a concentration that was shown under "Range Finding" to yield a constant rate of product formation for 30 min. Place the tubes containing substrate and the tube with the diluted enzyme in a 37° C water bath and incubate for a few minutes (but no longer than 5 min) for them to come to temperature. Then add 1.0 ml of the diluted enzyme to the pH 8.0 tube, mix, and *immediately* transfer a 0.5 ml aliquot of the reaction mixture to a test tube containing exactly 5.5 ml of 0.1 M sodium hydroxide. This sample is the zero time control. It is not necessary to take a zero time sample at more than one pH value. To the remaining substrate tubes add 1.0 ml of the diluted enzyme and mix. After exactly 30 min of incubation at 37° C take 0.5 ml samples from each tube and transfer them to tubes containing 5.5 ml of 0.1 M sodium hydroxide. Remove the reaction tubes from the bath, allow them to cool to room temperature and then determine the exact pH of each mixture with the glass electrode. Determine the *p*-nitrophenol content of the alkaline reaction mixture samples by measuring their *A* at 405 nm.

Prepare a plot of phosphatase activity (μmoles *p*-nitrophenol formed/hr) versus pH. What is the pH optimum for the wheat germ phosphatase?

Determination of the Michaelis Constant for *p*-Nitrophenylphosphate

Prepare a series of tubes containing 0.05, 0.1, 0.2, 0.5, and 1.0 ml of 0.025 M NPP. Add 2.0 ml of 0.1 M citrate buffer (pH 4.8) to each and sufficient water to yield a final volume of 4.5 ml. Prepare a dilution of stock phosphatase in 0.1% bovine serum albumin that was shown under "Range Finding" to yield linear formation of *p*-nitrophenol for 30 min. Incubate the substrate tubes and the tube of diluted enzyme for a few minutes in the 37° C water bath. At timed intervals add 0.5 ml of diluted enzyme to each tube. Mix and *immediately* remove a 1.0 ml sample (zero time sample or control) from each reaction mixture and then add the zero time samples to tubes containing 5.0 ml of 0.1 M sodium hydroxide. Sample each tube (as before, 1.0 ml aliquots, etc.) again after exactly 10-min incubation with enzyme. Repeat the sampling again after 20-min incubation. Perform *p*-nitrophenol determinations as usual. Use each zero time control tube as the blank for the corresponding tube that contained enzyme. Calculate the μmoles of *p*-nitrophenol liberated by the enzyme at each substrate concentration and time. Calculate the reaction velocity (μmoles of *p*-nitrophenol formed/ ml of reaction mixture/10 min) at each substrate concentration. Do the determinations at 10 and 20 min agree? If not, what should you do? Prepare a Lineweaver-Burk plot of your data (see page 79), expressing NPP concentration in moles/liter. Determine the V_{max} and the Michaelis constant for NPP, expressing each in appropriate units.

Inhibition by Inorganic Phosphate

Repeat the experiment for determination of the Michaelis constant, with the modification that 0.2 ml of 0.05 M sodium phosphate (pH 4.8) is added to each tube and the volume of water added to each tube is adjusted so that the final volume (before adding enzyme) is 4.5 ml. (Note: This experiment must be done with the same dilution of crude phosphatase and on the same day as the determination of the Michaelis constant. Why?) Calculate the reaction velocity at each substrate concentration as before. Plot a Lineweaver-Burk plot of these data on the same graph used for Michaelis constant determination. What type of inhibition do you observe? Calculate an inhibition constant from your data. Show your method of calculation clearly.

If time permits, determine the kinetic pattern of inhibition of phosphatase by 5 mM sodium fluoride, using a protocol like that given above. Discuss any differences you find between phosphate inhibition and fluoride inhibition.

WARNING
NaF is toxic! Use a propipette with NaF solutions.

DISCUSSION

The development of suitable assay conditions is an essential first step in routine assay, kinetic study or purification of an enzyme. Normally, we attempt to assay an enzyme under optimal conditions because the assay is most sensitive when the enzyme is most active. Hence, you might expect to assay an enzyme at its optimum pH with saturating substrate concentrations. Another reason for choosing such conditions is that the enzyme is usually (not always) most insensitive to small variations in pH at optimum pH. Furthermore, an enzyme is likely to be less sensitive to inhibitors and to form product at a constant rate for a longer time when substrates are saturating. However, it is not essential to assay an enzyme under

optimum conditions; there are situations in which you would not do so. For example, if the enzyme were unstable at its optimum pH, it would be preferable to assay it at a pH value at which it was more stable. Some enzymes are inhibited by excess substrate concentration, so you would normally avoid inhibitory levels of substrates. The *essential* points about enzyme assays are these: (a) you must perform control experiments to show that the assay response is due to enzyme action, and (b) you must show that the assay response is linearly related to time of assay and quantity of enzyme assayed under all experimental conditions employed. Do your results from this experiment meet these criteria?

EXERCISES

1. In the assay for β-galactosidase (Experiment 8), an assay similar to that used here was employed. In that case hydrolysis of the substrate yielded *o*-nitrophenol. However, a very different method of determining the relationship between absorbance and μmoles of product was used. Why was that method not used in the present case?
2. How could you determine whether part of the shape of your pH-activity profile was caused by enzyme inactivation?
3. What would be the effect on the value you determined for K_M and for V_{max} of doubling the concentr tion of enzyme used?
4. Would you expect phosphate to be a better inhibitor of acid phosphatase than sulfate? Why?
5. What is an Eadie plot? A Hofstee plot? How could they be used to determine K_M and V_{max} from kinetic data?

REFERENCES

Hollander, V. P. 1971. *Acid Phosphatases* (The Enzymes, 3d ed., Vol. IV). New York: Academic Press.

Joyce, B. K., and S. Grisolia. 1960. Purification and Properties of a Nonspecific Acid Phosphate from Wheat Germ. *J. Biol. Chem. 235:*2278.

Wharton, D. C., and R. E. McCarty. 1972. Enzymes: Partial Purification and Some Kinetic Properties of an Acid Phosphatase from Wheat Germ. *Experiments and Methods in Biochemistry.* New York: Macmillan.