yeast. Therefore, quantitation of FAD may be performed using this pDAO preparation. A calibration graph is prepared using standard solutions of $10^{-7}$ to $2 \times 10^{-6}$ M FAD, made by diluting a $10^{-3}$ M solution of commercial FAD. Duplicate assays are performed according to the standard procedure, with pDAO and FAD standard solutions as components B and C, respectively. The absorbance (blank where C = H2O) was plotted against FAD concentration as shown in Figure 1. Subsequently the students are able to measure the yeast FAD concentration in E2 using the kidney pDAO, reading from the linear part of the curve, where absorbance (ie initial reaction velocity) is proportional to FAD concentration. We use three dilutions of E2 (1, 2, 3-fold) to obtain velocity values in this linear part (Figure 1).

Discussion

By measuring the volume of E2 one can calculate the quantity of FAD and estimate the yield of FAD extracted from yeast knowing that 2 g of dried yeast were utilized (ie 5 ml extract) and that 1 g of wet yeast contains 18.5 mg FAD/kg.

The reaction rate is dependent on the concentration of active DAO in assays: thus the concentration of the DAO solution must be adjusted if necessary, in relation to its activity, so that a sufficient number of experimental velocity values are obtained in order to construct the linear part of the calibration graph. This graph is equivalent to the progression rate of versus active DAO concentration and therefore FAD must be a limiting factor.

A secondary reaction can take place: it is neither instantaneous nor very fast and does not interfere under the conditions described here. We have omitted catalase from the assay mixture with the aim of simplifying this latter; catalase may possibly need to be added in the case of longer reaction durations or in others preparations.

In a supplementary experiment we have utilized a crude preparation of DAO as an alternative, namely porcine kidney acetone powder. In this case we used ammonium sulfate precipitation for apoenzyme extraction and FAD separation. We also purified FAD from yeast extract by acid extraction and column chromatography (on Florisil with elution with pyridine.) This purification of FAD offers the possibility to measure it by its absorbance at 450 nm in comparison with enzymatically determined value. The yeast FAD content may also be determined.

References


An Experiment in Membrane Enzyme Purification with Triton X-114: Avocado Pear Polyphenoloxidase

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Introduction

Polyphenol oxidase (PPO), or $\alpha$-diphenol oxygen oxidoreductase (EC 1.10.3.1) catalyzes the oxidation of $\alpha$-diphenols to $\alpha$-diquinones as well as the $\alpha$-hydroxylation of monophenols. The physiological function of PPO remains unknown. In fact, PPO functions as a phenol oxidase in vivo only in senescent or damaged cells, being responsible for the enzymatic browning which occurs in fruits and vegetables when they are damaged. In healthy green tissues PPO exists in a latent form on the thylakoid membrane and it is not involved in the synthesis of phenolic compounds; in the functional chloroplast, PPO may be involved in some aspects of oxygen chemistry. The latent enzyme may be activated by several agents; one of these is trypsin. Nonionic detergents are widely used for the solubilization of membrane proteins. A diluted solution of Triton X-114 exhibits a cloud point at about 20°C, that is, a solution of the nonionic detergent Triton X-114 which is homogeneous at cold room temperature, separates in an aqueous phase and a detergent phase above 20°C. This property can conveniently be used for the isolation and further purification of membrane proteins.

Justification of the Proposed Laboratory Practical

Students will learn strategies and reasoning in protein purification. The choice of ripe fruit PPO is justified as follows: (i) PPO is a membrane-bound enzyme that may easily be partially purified in one step by compartmentation in Triton X-114, (ii) PPO may be purified as a latent enzyme, (iii) latent PPO may be stored at $-20\degree C$ without loss of activity for many months; it also maintains its activity at 4°C for several days at least, and (iv) the starting material is relatively cheap and easy to obtain. The choice of the purification procedure is justified for the following reasons: (i) Compartmentation in Triton X-114 makes it possible to isolate the latent PPO in the aqueous phase, (ii) the complete procedure may be carried out in a single experimental session, and (iii) this procedure removes phenolics and chlorophyll in one step without chromatography and avoids aggregation and inactivation of the enzyme during purification.

Latent PPO Extraction and Purification in Triton X-114

The procedure to purify PPO from avocado fruit is the Triton X-114 method firstly described for grape berries by Sánchez-Ferrer et al. Ripe avocado pear flesh (25 g) is added to 25 ml of 0.1 M sodium phosphate buffer, pH 7.3, containing 10 mM sodium ascorbate and homogenized by using a mechanically operated Potter (it can be also homogenized by hand). The homogenate is filtered through muslin and centrifuged at 4000 g for 15 min. The pellet is extracted with 20 ml of 1.5% (w/v) Triton X-114 in phosphate buffer for 30 min at 4°C. After ultracentrifugation at 60 000 g for 15 min, the supernatant is subjected to temperature...
phase compartmentation by adding Triton X-114 at 4°C, so that the final detergent concentration is 4% (w/v). The mixture is kept at 4°C for 15 min and then warmed to 30°C. After 10–15 min the solution became spontaneously turbid and partitioned in an upper aqueous phase, and a lower detergent-enriched phase. After centrifugation at 5000 g for 10 min at room temperature, the two phases are carefully separated and stored at 4°C or −20°C until enzyme assay.

**Enzyme Assay**

PPO is assayed by using DL-DOPA as substrate and recording the time course in a double-beam spectrophotometer at 480 nm. The sample cuvette contains 5 mM DL-DOPA, 0.1 M phosphate buffer, 100 units of catalase, and 0.125 ml of extracted PPO in a final volume of 1 ml; the blank cuvette lacks of PPO.

**Activation by trypsin**

The process of activation by trypsin is completed in five minutes at 37°C using 2000 units/ml. The trypsinized PPO shows an activity several times higher than the latent PPO.

**Suggestions for a Second Day of Practical Work**

If a second session of 4-6 hours is possible, it is suggested that the students perform an electrophoresis and carry out a more detailed enzyme characterization.

A further advantage of PPO is that it maintains its activity after SDS-PAGE. Electrophoresis is carried out as described by Angleton and Flurkey\(^1\) for 4–5 h at room temperature. Afterwards gels may be stained for PPO activity in 10 mM acetate buffer (pH 5), containing 5 mM DL-DOPA.

As the SDS-PAGE is running, enzyme characterization may be carried out. First, a classical kinetic study, with determination of affinity constant, inhibitor studies, and product inhibition. Afterwards a study of the effects of pH and temperature on enzyme activity can be performed.

**Discussion**

It is recommended that the proposed practical class experiment be carried out in small groups (2–3 students). The experimental procedures are easy enough and PPO activity is "hard" enough to ensure a successful work. A session of discussion of the theoretical background and the strategy to be carried out previously to the laboratory sessions guarantee an easy assimilation of important concepts in protein biochemistry.

Two of the authors (R G V and A G H) were students in a General Biochemistry Course during the optimization of the practical. They cooperated actively in this optimization and in this way they learned to use a range of basic biochemical techniques.

**References**


**Heteroionic Exchange in Bone Powder: A Laboratory Experiment**

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**Introduction**

The importance of the binding fluoride ion to dental enamel has long been recognized. It has been observed, for instance, that the arithmetic mean of the index of caries in a population was directly related with the content of fluoride in the drinking water of each region or city studied. The addition of fluoride, in the proportion of 1 ppm, to the drinking water in those regions where the content of fluoride was much lower resulted in a considerable reduction in the incidence of caries.

In those regions where the fluoride content of drinking water was higher (about 5 ppm or higher), the appearance of dots (undesirable in humans due to its ugly aspect) as well as a certain fragility in the enamel caused some concern, although a resistance to the development of caries was also noted.

These events have received a great deal of publicity, especially since they produced a great development in the techniques employed to combat dental caries so common in children.

Interest in this field is increasing and the use of fluoride is being studied from many aspects. New methods are being designed for determination of fluoride in water, the influence of the composition of the solution on the properties and features of the carbonate apatite formed, the influence of fluoride in the evolution of osteoporosis and other diseases, etc. In the dental field, of course, studies continue and have been published, i.e., the influence of several fluorides on erupted and non-erupted teeth.

The phenomena that take place are not so readily taught due to their complexity and the chemical knowledge required; and while many people do know that the resistance to acid dissolution is due to the transformation of hydroxyapatite in fluorapatite, very few know how to demonstrate this fact. The purpose of this simple laboratory practical described below is to make visible to students the chemical reactions occurring and to encourage them to think about the true reaction and other possible reactions that could explain the results obtained in this practice.

**Experimental**

**Materials required**

1. Test tubes 16 x 150 mm
2. Sodium fluoride (50 mg)
3. Defatted and dry bone (50 mg)
4. Deionized water (10 ml)
5. Phenolphthalein indicator (2 drops)

**Procedure**

Add the phenolphthalein indicator to the water and mix. Then add 5 ml of the mixture to each of the other tubes one containing NaF, the other containing bone powder and mix thoroughly. Transfer the contents of the tube with sodium fluoride to the tube with bone powder. Wait 2–4 minutes and observe.

1. The addition of phenolphthalein to water does not produce any change of colour because the pH of water is lower than 8.0.
2. The addition of water with phenolphthalein to sodium fluoride or bone powder does not produce any colour change because the pH of the resulting solutions is lower than 8.0.
3. The mixture of sodium fluoride and bone powder produces a red colour due to the exchange of fluoride ions with the hydroxyl ions in bone powder. The hydroxyl ions added to the solution increases the value of pH in the solution and as a consequence, the phenolphthalein turns red.

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