A simple laboratory experiment for the teaching of the assay and kinetic characterization of enzymes

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A Simple Laboratory Experiment for the Teaching of the Assay and Kinetic Characterization of Enzymes

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Introduction

In our Faculty, Biological Chemistry is one of the obligatory courses in the Pharmacy course. Enzymology is one of the more important subjects in this course because of the significant number of pharmacological drugs acting on different enzymes. In the teaching of Biological Chemistry to Pharmacy students, we found difficulties in introducing enzymology concepts, such as the significance of enzyme activity, ways of measuring it by continuous and discontinuous methods, and the importance of knowing the linearity conditions for an enzymatic reaction. We looked for a laboratory practical to include all of the above subjects, that, at the same time, could be put on as a relatively inexpensive laboratory class, considering our limitations in equipment (ie a visible, non-UV spectrophotometer). This work reports on the laboratory section thus prepared, which includes the measurement of the activity of two enzymes (*urease* and *uricase*), one using a continuous and the other a discontinuous photometric method.

Urease (EC 3.5.1.5) catalyzes the practically irreversible hydrolysis of urea:

$$\text{urea} + H_2O \longrightarrow CO_2 + 2NH_4^+$$

This nickel-containing enzyme is found in plants, fungi and bacteria and has the historical interest of being the first enzyme to be crystallized.[1] The use of microencapsulated urease is being developed as a useful system to maintain urea levels in the blood of patients suffering alterations in kidney function.[2] Urease is utilized for diagnostic purposes, in the determination of urea in biological fluids. The enzymatic, colorimetric, endpoint method for urea measurement is based on urease-catalyzed hydrolysis of urea followed by the quantitation of ammonium by reaction with hypochloride and phenol producing indophenol, which can be measured photometrically.[3]

Uricase (EC 1.7.3.3) is a copper-protein found in animal tissues (those that do not eliminate intact uric acid) and yeast (for a review see ref 4), catalyzing the oxidation of uric acid as follows:

$$\text{uric acid} + O_2 + 2H_2O \longrightarrow \text{allantoin} + CO_2 + H_2O_2$$

Interestingly, uricase is absent in man and higher primates though when its gene is still present (but not expressed) in these organisms.[5] The enzyme is utilized for the quantitative determination of uric acid in pathological situations caused by increased production of this compound (gout).[5] Measurement methods are supported by the determination of allantoin at 290 nm,[6] or by coupling the uricase reaction with peroxidase (alone or plus other enzymes).[7,8] In one of these methods, H_2O_2 produced in the uricase reaction is cleaved by peroxidase and this coupled to the formation of quinone-imide dye from 3,5-dichloro-2-hydroxybenzenesulfonate and 4-aminoantipyrine.[7,8]

In our laboratory experiment, urease is measured as an example of discontinuous enzymatic assay, whereas uricase coupled to peroxidase is determined by a continuous method. Both enzymes are measured colorimetrically and their characterization includes determination of the linearity conditions for the reaction, kinetic parameters and the effect of different substrate analogs.

Experimental

Commercial diagnostic kits for urea and uric acid were utilized for the laboratory class. We employed kits kindly donated by *GT Laboratorio SRL*, a chemical company which produces reagents for clinical diagnostic, located in Rosario (Argentina). These kits are similar to those provided by other chemicals suppliers (ie Sigma Chemical Co, St Louis, MO, USA). Substrates for the enzymatic reactions were from the respective standard solutions of diagnostic kits, or alternatively prepared from chemicals of high quality (analytical grade). Urea solutions were prepared in water and concentration determined using the same enzymatic endpoint method.[3] Uric acid solutions were freshly prepared in 0.01 M NaOH and concentration determined spectrophotometrically at 293 nm.[6] Hypoxanthine, xanthine, allopurinol and oxypurinol were freshly prepared and their concentrations determined spectrophotometrically.[9,10]

Assay of urease activity was carried out at 30°C. Standard assay medium contained 0.05 ml of 40 mM Pi buffer, pH 7.5; and 0.1 ml of urea solution (different concentrations, as stated). The enzymatic reaction was initiated by addition of 0.05 ml of urease (adequate dilution in water of the partially purified suspension provided with the kit), incubated the time specified in each case, and stopped by the addition of 2.4 ml of color reagent (added as specified in the protocol provided with the kit). After 30 min, absorbance at 530 nm was measured. When necessary, assays were performed in the presence of different concentrations of thiourea.

Alternatively, urease reaction can be measured under identical conditions but using purchased chemicals and reagents instead of manufactured kits. Urease to be utilized is a protein partially purified from jack beans and the color developing reagent is composed of: *reagent A* 0.5 M phenol and 0.4 mM sodium nitroferricyanide(III); *reagent B* 0.67 M NaOH and 45 mM sodium hypochlorite. The above stated addition of 2.4 ml of color developing reagent to stop the urease reaction is performed by adding 0.2 ml of *reagent A*, then 0.2 ml of *reagent B* and, after 5 min of incubation, 2 ml of water.

Uricase activity was measured at 490 nm, at 30°C, and in a final volume of 1.5 ml. The assay medium contained the mixture of color developing reagents (specified by the protocol provided with the kit) in Pi buffer, pH 7.5; uricase (adequately diluted in Pi buffer plus 5% glycerol); an excess of peroxidase (5 Units); and the specified concentrations of uric acid. The reaction was initiated by the addition of the later compound and continuously measured at different time intervals (if available a recorder may be connected to the photocolorimeter to continuously register the reaction). Assays in the presence of different concentrations of xanthine, hypoxanthine, allopurinol or oxypurinol were carried out to analyze the effect of these compounds on the enzyme activity.

The kit for uricase measurement can be conveniently replaced by using chemicals purchased separately. Thus, uricase from *Arthrobacter globiformis* or from porcine liver may be used, with peroxidase from horseradish. Color reagent for uricase

reaction needs of two solutions: *A* 30 mM 4-aminoantipyrine; and *B* 12 mM 3,5-dichloro-2-hydroxybenzenesulfonate, 12 mM 2,4-dichlorophenol in 0.7 M Pi buffer, pH 7.5. The assay medium for uricase is then prepared by mixing 0.15 ml of *solution A*, 0.15 ml of *solution B*, uricase and peroxidase as specified above, and water to a final volume of 1.5 ml.

Kinetic parameters (K_m and K_i values) were determined by double-reciprocal plots (1/*v* versus 1/[substrate]) and Dixon plots (1/*v* versus [inhibitor]) of the velocity data.[1]

Results and Discussion

The use of a continuous method to measure enzyme activity allows one optimally to observe conditions of linearity for the catalyzed reaction. Fig 1 shows the time–course for uricase measured at different substrate levels. Several conclusions can be made from Fig 1: (i) the time interval over which the reaction follows linearity is dependent on substrate concentration; (ii) under the specified assay conditions incubation time of 3 min or lower should be utilized if enzyme activity is measured by using a single point determination at different (and unknown) substrate concentrations. In this example, the best determination of the enzymatic reaction velocity is made by extrapolating the time–course curve to zero time. In this way, straight lines, shown in Fig 1 (dashed) for the two extreme values of substrate concentration (0.007 and 0.19 mM), are obtained and thus velocity values calculated; (iii) to use the assay for uric acid measurements, times where no increase (or very low) of absorbance occurs (at the different substrate concentrations) should be utilized. In Fig 1 this condition is marked by the vertical line at 60 min.

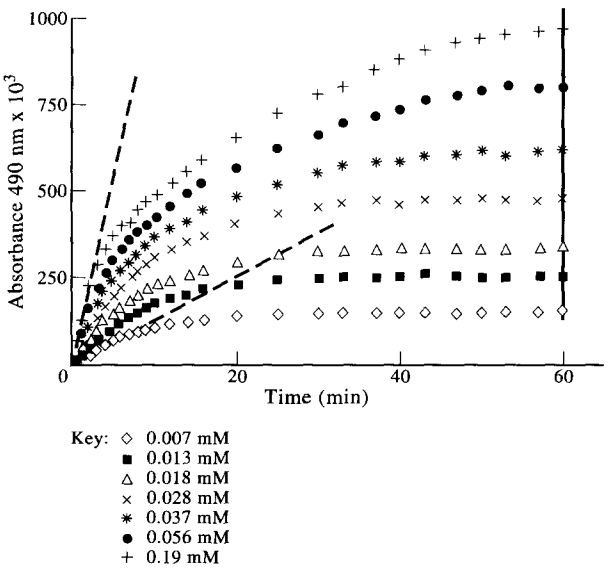

Figure 1 Time-course of the reaction of the uricase, assayed at the concentrations of uric acid shown in mM. Dashed lines indicate extrapolation to zero time to calculate initial velocity at the uric acid concentrations of 0.007 and 0.19 mM

Consequently, from Fig 1 two graphs may be derived. One determining velocity values for the enzyme activity (those calculated as showed by dashed lines, for each uric acid concentration utilized), thus resulting in the substrate saturation curve showed in Fig 2. From these data, a K_m value of 0.03 mM for uric acid was determined (Table 1). This value is in agreement with those determined for uricase from different sources.[4] The second graph is obtained from absorbance values at 60 min (vertical line in Fig 2) as a function of uric acid concentration, which produces a calibration curve for the acid measurement shown in Fig 3. As shown, under our experimental conditions, the response to uric acid is linear over the range 0–7.5 nmol. It is worth to emphasizing that from the data in Fig 1 it is possible to visualize the different working conditions necessary to determine a kinetic parameter for uricase or to use the enzyme to estimate uric acid concentration in a sample.

Fig 4 shows the time–course for the reaction catalyzed by urease at 0.1 and 10 mM urea. It is interesting to note that the necessity of utilizing a discontinuous method for this determination turns very critically on the establishment of the linearity conditions for activity. In this case, it is very important to define clearly linearity conditions when kinetic parameters of the enzyme are to be determined, since it is not possible to obtain a continuous plot of absorbance versus time. As will be seen from

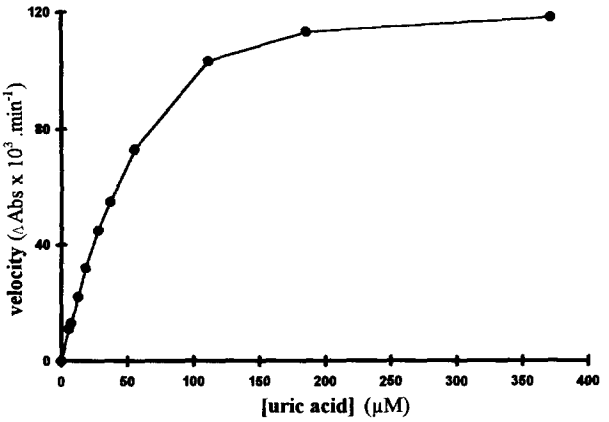

Figure 2 Substrate saturation curve for uricase. Velocity values were calculated from Fig 1 and plotted versus the respective uric acid concentration

Table 1 Kinetic parameters for the interaction of uricase and urease with their substrates and with substrate analogs behaving as inhibitors

Enzyme	*Compound*	*Effector type*	K_m *(mM)* *Kinetic parameter*	K_i *(mM)*
Uricase				
	Uric acid	Substrate	0.031	
	Xanthine	Inhibitor (C)		0.033
	Hypoxanthine	Inhibitor (C)		0.84
	Oxypurinol	Inhibitor (C)		1.20
	Allopurinol	Inhibitor (C)		2.10
Urease				
	Urea	Substrate	0.98	
	Thiourea	Inhibitor (C)		12.5

(C): competitive inhibitor

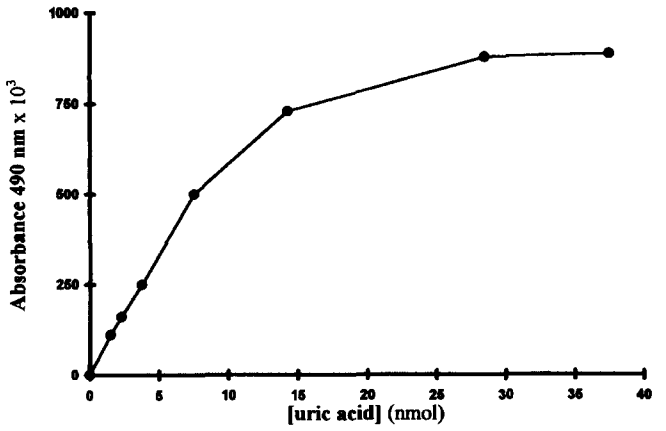

Figure 3 Calibration curve for the measurement of uric acid using uricase assay. Absorbance values at 60 min, obtained from Fig 1, were plotted against nmol of uric acid in the assay medium

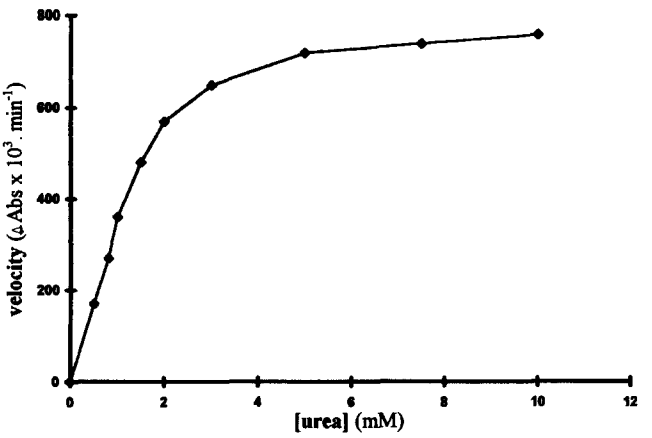

Figure 5 Substrate saturation curve for urease. Velocity values were obtained by single point determination of activity (2.5 min) and plotted versus the corresponding urea concentration

Fig 4, to estimate the K_m for urea, and working in a substrate concentration range 0.1–10 mM, incubation times of 3 min (or lower) must be utilized. Under these conditions saturation curve for urea was obtained as shown in Fig 5, and a K_m value of 1 mM was determined. On the other hand, if the urease reaction is employed for the determination of urea in biological fluids, it is important to incubate for longer times, until the absorbance values remain constant or changes only slightly which will be 6 min (or longer) in the example shown in Fig 4.

Further characterization of uricase was carried out by studying the effect of different substrate analogs when present in the assay medium. As shown in Table 1, xanthine, hypoxanthine, oxypurinol and allopurinol were competitive inhibitors of the enzyme in a decreasing order of inhibitory effect (compare K_i values in Table 1). Of interest is the effect of allopurinol and oxypurinol on uricase, since the former is a drug utilized in the treatment of gout and it is metabolized by xanthine oxidase to oxypurinol in the patient. Thus, when uric acid measurement is necessary in the blood of a person suffering from gout some underestimation could be caused by allopurinol medication. However, a comparison of reported values of allopurinol and oxypurinol (0.01–0.1 mM)[11] in blood of medicated patients with K_i values (1–2 mM) determined in our study, together with the low K_m value of the enzyme for the substrate (0.03 mM) and normal levels of uric acid in blood (<0.36 mM), suggest that no significant interference occurs in uric acid dosage, because the low affinity of uricase towards the competitive inhibitors.

The inhibitory effect of uric acid analogs shown in Table 1, is in agreement with the reported properties of uricase.[4] None of the analogs was effective as a substrate, thus indicating the high specificity of the enzyme with respect to uric acid.[4] An interesting conclusion may be reached if results in Table 1 are analyzed in relation to the structure of the inhibitors shown in Fig 6: the -OH group in carbon 2 is important for the binding of the substrate to the enzyme. The interaction of this -OH group

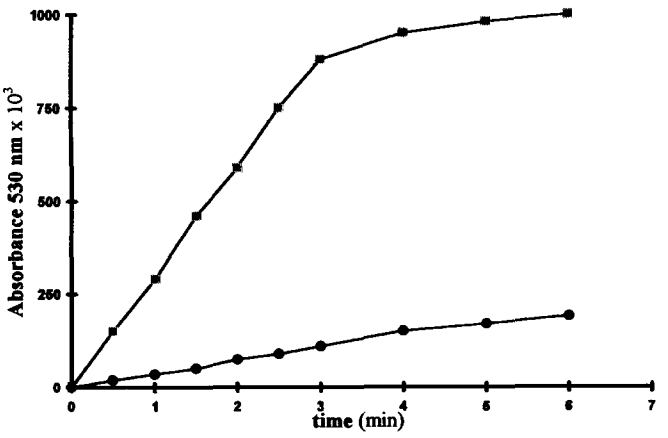

Figure 4 Time–course of the reaction of urease, assayed at 0.1 (●) and 10 (■) mM urea

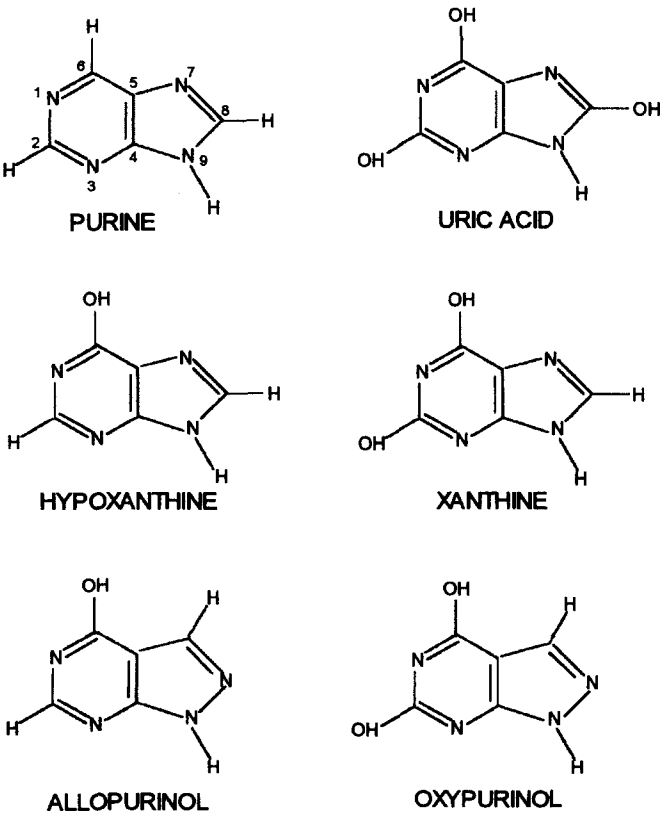

Figure 6 Structure of purine, uric acid and the different analogs analyzed in this study as effectors of uricase

with a basic residue in the active site of the protein,[4] explains the higher K_i value for hypoxanthine compared with xanthine, and the lower inhibitory effect of allopurinol with respect to oxypurinol.

On the other hand, Table 1 also shows that thiourea behaved as an inhibitor of urease, competitive with respect to urea, and with a K_i of 12.5 mM. This indicates that the affinity of urease for the binding of urea is significantly higher than for the analog thiourea, as deduced from the order of magnitude difference in the kinetic parameters determined. The later, together with the fact that thiourea is not effective as a substrate agrees with the very high specificity of urease for urea.[12]

Concluding remarks

The laboratory practical we have developed is quite simple to put on and in our hands is very useful for teaching the understanding of enzyme assays and enzyme characterization. Worthy of mention are: (i) the relatively low cost of the materials necessary, which may be obtained and prepared from commercial kits (or alternatively prepared from purchased chemicals) and require only simple equipment; (ii) the good examples represented by the assays in terms of the understanding of measurement of enzyme activity by continuous and discontinuous methods, with the corresponding importance for finding linearity conditions in each case; and (iii) the possibility to analyze the different working conditions, depending whether the assays are to be used for the determination of kinetic parameters of the enzymes or to quantify their substrates in biological fluids.

The laboratory practical is also useful to further characterize the enzymes, such as to analyze the effect of substrate analogs. This analysis is of interest to investigate the specificity of the enzymes and to emphasize the importance of substrate structure in its interaction with the protein. The section could also be utilized to carry out other studies on enzyme characterization: ie determination of pH and/or temperature optima, which should be prepared on the basis of the conditions described in the present work.

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A Demonstration of Genomic DNA Profiling by RAPD Analysis

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Introduction

Characterisation of genomic DNA through identification and determination of random polymorphic markers has proved a powerful application of DNA technology over the last decade. It has been used in applications as wide ranging as paternity testing and forensic science, through investigation of genetic disease genes and identification of the sources of plant and animal products, to ecological population studies. This makes it an interesting and relevant subject for a practical class in a range of courses.

DNA profiling techniques were developed using purified DNA which was digested with restriction enzyme(s), Southern blotted and then probed with randomly picked DNA probes. Using this approach, sequence polymorphisms at restriction enzyme sites, and insertions or deletions between sites, could be identified and used for genome characterisation. More recently, probes have been selected to identify polymorphisms in specific types of DNA (usually repetitive sequence DNA). This methodology, in which heritable differences between genomes in the location of sites for a restriction enzyme are identified and used, is called restriction fragment length polymorphism (RFLP) analysis. Its main drawbacks are that it requires microgram quantities of relatively pure genomic DNA, and it involves Southern blotting and probe hybridisation and detection. This makes the analysis time-consuming and potentially costly.

Since its introduction in 1985 the polymerase chain reaction (PCR) has become a central technique in many areas of DNA manipulation and analysis. Its usefulness is related to its selectivity for the target DNA sequence, the large number of copies made from the sequence, the rapidity of the procedure and the ability of the system to amplify DNA from a relatively impure preparation.

PCR with random sequence primers offers a fast approach to genome profiling. The most popular technique of this type is called Random Amplification of Polymorphic DNA (RAPD).[1] The primers used are normally 10 bases in length. This is short enough for annealing to occur at a number of positions in the genome. When annealing of the single primer in the reaction occurs on opposite strands of a stretch of DNA which is short enough to form a template for PCR, a product will be formed. It is probable that this situation will occur at several points in the genome, giving rise to several PCR products which can be resolved by gel electrophoresis to give a characteristic pattern. Small sequence differences at a point of primer annealing will prevent annealing and amplification from that part of the genome. Additionally, insertions or deletions between primer annealing positions will lead to a change in the length of the PCR product. Such changes are heritable, and product profiles can be used in a similar way to RFLPs.[1,2] The advantage of this approach over RFLPs is that because of the amplification aspect of the PCR, much less DNA is required for analysis, and contamination of the DNA with other cell components is less likely to affect the analysis.[2,3] RAPD analysis can be used as a