Student Code: _____

20th INTERNATIONAL BIOLOGY OLYMPIAD

12th – 19th July, 2009

Tsukuba, JAPAN

PRACTICAL TEST 2

BIOCHEMISTRY
生物化學
Total Points: 100
Duration: 90 minutes

Dear Participants,

- In this test, you have been given the following 2 tasks:

  Task 1: Measurement of acid phosphatase activity (70 points)

  Task 2: Protein determination (30 points)

  本實作包括兩部分：

  第 1 部分：酸性磷酸酶活性的測定 (70 分)

  第 2 部分：蛋白質的測定 (30 分)

- **You must write down your results and answers in the ANSWER SHEET. Answers written in the Question Paper will not be evaluated.**
- Please make sure that you have received all the materials and equipment listed for each task. If any of these items are missing, please raise your hand.
- At the end of the test, put the Answer Sheet and Question Paper in the envelope. The supervisor will collect this envelope.
- This series of practices are time consuming. You will need to be well organized and work quickly to complete the five tasks.
- 你必須把結果及答案寫在答案紙上。寫在考卷上的答案不給分。
- 確定材料與器材是否齊全，若有缺失，請舉手告知監試人員。
- 本實作結束後，請將答案紙及考卷一起放入信封中，待監試人員收回。
- 本實作相當費時，你的操作必須很有組織而且快速，才能完成五個部分。

Good Luck!!

How to use the spectrophotometer 光電比色計的使用方法

1. The screen of spectrophotometer (Shimadzu UVmini-1240) must show 400 nm (Fig. 1). If not, raise your hand. ABS value shown may not be 0.000.
2. Fill a plastic semi-micro cuvette with distilled water (DW) at least up to the shoulders inside (Fig. 2)
3. Insert the cuvette into the cuvette holder of the instrument, with the transparent surfaces facing to the left and right (Fig. 3).
4. Shut the lid (Fig. 4).
5. Press 'AUTO ZERO' button (Fig. 5). By this manipulation, the instrument regards the level of absorbance by the cuvette plus water as zero. This will be used as a blank control for the rest of the rest of this experiment.
6. Now, you are ready to measure absorbance of samples.
7. Replace the water with a sample solution and read an ABS value after the lid is shut. The absorbance is caused by solutes in the sample solution.
8. You do not have to wash the cuvette after every measurement, if you measure a series of samples from the dilute to the concentrated.

1. 光電比色計的螢幕必須顯示 400 nm (如 Fig. 1 所示），若否，舉手通知監試人員。但螢幕上的吸光度(ABS 值）可以不是 0.000.
2. 在光電比色管中裝入蒸餾水(DW），至少裝到 ” shoulders ”處(如 Fig. 2 所示）。
3. 將光電比色管放入光電比色計中(如 Fig. 3 所示）的比色管放置處（cuvette holder），透明面須朝向左右兩側。
4. 關上蓋子(如 Fig. 4 所示）。
5. 按下'AUTO ZERO'的按紐(如 Fig. 5 所示），儀器將以此吸光值視爲標準零值。本實驗將以此爲標準零值（blank control)。
6. 接下來，你可以開始測量其他樣本的吸光值。
7. 將蒸餾水換成其中一種樣本溶液，操作同上，並讀取其吸光值。
8. 如果你的序列稀釋的樣本，是依濃度低往濃度高的樣本依序測量，則在每次測量後，你將不須清洗光電比色管。

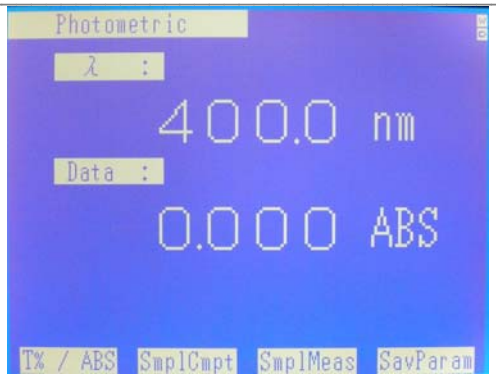


Fig. 1

Fig. 2

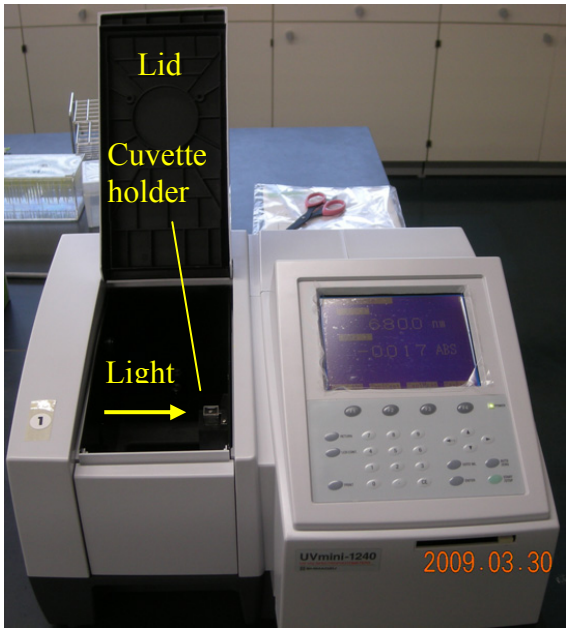


Fig. 3

Fig. 4

Fig. 5

Introduction

Acid phosphatase liberates phosphate ions from phosphorylated molecules under acidic conditions. The purpose of this experiment is to determine the specific activity of the acid phosphatase. You will measure activities of the acid phosphatase using a crude extract from potato in Task 1, and determine a protein concentration of the crude extract in Task 2. Specific activity, which is the activity per unit time per unit weight of protein, is obtained from Tasks 1 and 2. Specific activity is an index of purity; it increases as the enzyme is purified.

酸性磷酸酶在酸性環境中，可以將磷酸根離子從磷酸化的分子中釋出。本實驗的目的在判定酸性磷酸酶的活性。在第 1 部分，你將利用馬鈴薯的粗萃液來測量酸性磷酸酶的活性；在第 2 部分，你將判定粗萃液中所含蛋白質的濃度。活性在此是指由第 1、2 部分所獲得之每單位時間、每單位量的蛋白質。此活性可作爲酶純度的指標，當酶的活性愈高，則代表酶的純度愈高。

Caution 注意

1. You will be handling small amounts of toxic substances (*p*-nitrophenol and NaOH).You can choose to wear disposable gloves and safety goggles in the experiments if you like.
2. In calculations where answers to previous questions are needed, partials marks will be given if calculated formulas are correct, even if answers are incorrect.

1. 你將會使用少量的有毒物質（*p*- nitrophenol and NaOH），如有需要，你可穿戴丟棄式手套及護目鏡。
2. 承上述的實驗結果來進行計算時，只要你計算公式正確，即使答案錯誤，仍可有部分得分。

Materials and Equipments 材料與器材	Quantity
1. Spectrophotometer 光電比色計	1
2. Micropipettes (P1000) 微量吸管	2
3. Micropipettes (P200) 微量吸管	1
4. Tips (one box each for P1000 and P200) 微量 Tips	2
5. Plastic cuvette 塑膠光電比色管	1
6. Test tube holder that accommodates 6-1 to 6-6 可容納六根試管(6-1 to 6-6) 的試管架	1
6-1. Crude extract of acid phosphatase (4 ml in a 15-ml plastic tube, labeled “1X” enzyme) 酸性磷酸酶的粗萃液（4 ml，置於 15-ml 的塑膠試管中並標示 1 倍“1X”的酵素）	1
6-2. 0.5 M Na acetate buffer (pH 5.6) (2 ml in a 15-ml plastic tube) 0.5 M 醋酸鈉緩衝溶液(pH 5.6) （2 ml，置於 15-ml 的塑膠試管中）	1
6-3. 5 mM pNPP (4 ml in a 15-ml plastic tube)	1
6-4. 0.5 M NaOH (8 ml in a 15-ml plastic tube)	1
6-5. 3% NaCl (10 ml in a 15-ml plastic tube)	1
6-6. Test tubes (Glass)	6

Task 1 (70 points)

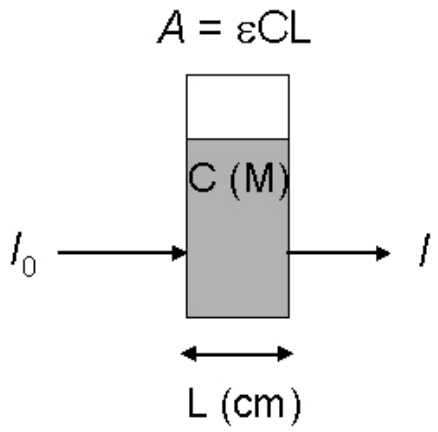
Measurement of acid phosphatase activity

酸性磷酸酶活性之測量

The activity of acid phosphatase is measured by an enzymatic reaction that converts *p*-nitrophenyl phosphate (pNPP) to *p*-nitrophenol (pNP), liberating phosphate. The product, pNP, absorbs light whose wavelength is 400 nm with an absorption coefficient*($\varepsilon_{400\ nm}$) of 19000 $M^{-1}cm^{-1}$ at extremely alkaline pH. Reaction mixture for an acid phosphatase is slightly acidic. Thus, it must be alkalinized for quantification of pNP. In Task 1, you will measure a time course of the reaction and obtain absorbance change per minute that is caused by 1 ml of crude extract. The absorbance change is converted to concentration change by using $\varepsilon_{400\ nm}$. Then, you will calculate a mol number of pNP molecules produced during the reaction by multiplying the concentration change by a volume of sample that is subjected to the measurement of absorbance.

酸性磷酸酶活性的測定原理，在於此酶可催化將 *p*-nitrophenyl phosphate (pNPP) 轉化成 *p*-nitrophenol (pNP)的酵素反應過程。此反應會釋出磷酸根，且其產物 pNP 在極度鹼性的環境中，於 400 nm 的吸收光譜下的吸光係數（absorption coefficient*($\varepsilon_{400\ nm}$)）爲 19000 $M^{-1}cm^{-1}$。進行酸性磷酸酶的反應時，其反應混合液具有微酸性，故若需量化 pNP，必須將混合液轉成鹼性。在實驗 1，你將測量加入 1 ml 的粗萃液後之反應的時間歷程及每分鐘所得之吸光值改變。利用$\varepsilon_{400\ nm}$可將此吸光值改變換算成濃度改變的數值，然後，再將換算得的濃度數值乘以你當初測定的樣本體積，即可計算出反應中 pNP 分子的莫耳數。

*What is an absorption coefficient？什麼是吸光係數？



A, absorbance 吸光值

ε, absorption coefficient (M^{-1} cm^{-1}) 吸光係數

C, concentration (M=mol $litre^{-1}$) 濃度

L, light path length (cm) 比色管中，光所經過的路徑長度

I_0, intensity of incident light 入射光之強度

I, intensity of transmission light 透射光之強度

Absorbance (*A*) is a physico-chemical property of solution that expresses to what extent a solute absorbs light at a specific wavelength. Absorbance is in proportion to concentration (C) and light path length (L). The constant in the equation is a value characteristic to the solute, and is termed the absorption coefficient (ε). Thus, the relationship is formulated as $A = \varepsilon$ C (M=mol $litre^{-1}$) L (cm). Absorbance can be converted to concentration, since ε is given and L is 1 cm in this experiment. The dimension of ε is M^{-1} cm^{-1}, because absorbance is an absolute number without units.

吸光值是溶液的一種物理-化學特性，其顯示溶劑在某種特定光波長下所能吸收光的程度。吸光值與濃度(C)及比色管中光所經過的路徑長度(L)成比例。公式中的常數(ε)代表溶劑的特性，稱爲吸光係數(ε)。因此，以此 $A = \varepsilon$ C (M=mol $litre^{-1}$) L (cm)公式表示這些因子之間的關係。由於 ε 是已知值，且 L 在此實驗中是 1 cm，故吸光值可轉換成濃度。ε 的單位爲 M^{-1} cm^{-1}，因爲吸光值是沒有單位的絕對值。

Two enzyme concentrations are to be examined in Task 1. Find the test tube on which '1X enzyme' is labeled, which contains a crude extract of acid phosphatase. Next, find the 15-ml tube that contains 3% NaCl and remove 1 ml of the solution so that the tube now contains 9 ml of 3% NaCl. Add 1 ml of the 1X enzyme solution to it by using a micropipette, which makes '0.1X enzyme' solution. Label the tube as '0.1X'. Next, find 6 empty test tubes. Label each tube with an enzyme concentration and a reaction time as follows.

在實驗 1，有兩種不同濃度的酶被檢測，選取標示 1 倍(1X)酶的試管，其內爲含有酸性磷酸酶的粗萃液。而後，從含有 3% NaCl 溶液的 15-ml 試管中取出 1 ml，此時試管中只剩 9 ml 的 3% NaCl，接著以微量吸管量取 1 ml 的 1 倍 (1X) 酶加入試管中，而得到 0. 1 倍(0.1X)酶，標示此試管爲 0. 1 倍(0.1X)。接下來，取 6 個空試管，標示每個試管之酶濃度與反應時間如下：

0.1X, 20 min
1X, 20 min
0.1X, 10 min
1X, 10 min
0.1X, 1 min
1X, 1 min

Q.1.1. (10 points) First, make an experimental schedule in order to perform all reactions, by describing start (○) and stop (●) signs for each reaction in the table in the Answer Sheet, allowing at least 1 min between the beginning of each reaction. An example for the reaction of '0.1x, 20 min' has been described in the table in the Answer Sheet.

首先規劃一個可以一次完成所有反應的實驗設計，在答案卷上針對每個反應，將你的實驗設計填入答案卷上 **Q.1.1.**的表格中，以(○)表示反應開始，(●)表示反應停止，實驗設計時，在各反應作用之間至少要有 1 分鐘的間隔，以方便操作。注意！答案卷 **Q.1.1.**表格中有一個範例，是反應'0.1x, 20 min' 的實驗設計方式，請依此範例，完成其他反應的實驗設計。

Q.1.2. (15+10 points) Perform the enzymatic reactions according to the protocol described below and the schedule you made in Q.1.1. Use a new pipette tip in every manipulation. Agitate a mixture by tapping a test tube immediately after an addition. After you perform all the reactions, measure A_{400} of the samples. Write the obtained values in the table in the Answer Sheet, and plot them in the graph. Please note that since water has been used as a blank, the line will not pass through 0 (zero) on Y-axis (origin).

根據下列所敘述的步驟，並配合你在上題(Q.1.1.)所列的實驗流程，在每次操作中，請用新的微量吸管頭(tip)；在每次加入藥品後，立即輕彈試管以混合溶液。當你進行所有的反應後，測定各樣本之 A_{400}(400 nm 光源下的吸光值)，將所得的結果填於答案卷的表格中，並根據此數據作圖。請注意:由於本實驗採用水當作標準零值(blank)，所以作圖所得的直線將不會通過 Y 軸的 0 點。

酸性磷酸酶活性測定的實際操作步驟如下：

Protocol for measurement of acid phosphatase activity

酸性磷酸酶活性之測定方法

1）Mix 0.12 ml of 0.5 M Na acetate buffer (pH 5.6) and 0.24 ml of 5 mM pNPP in a test tube. Start the reaction by adding 0.24 ml of an enzyme solution.

2）After the reaction times of 1, 10, and 20 min, respectively, stop the reaction by adding 0.6 ml of 0.5 M NaOH. NaOH stops the reaction and converts the pNP produced into a yellow-colored (A_{400}-absorbing) form.

3）After all reactions are stopped, measure A_{400} of the samples.

1. 在試管中加入 0.12 ml 的 0.5 M 醋酸鈉以及 0.24 ml 的 5 mM pNPP，混合後，再加入 0.24 ml 的酶溶液，開始反應。
2. 開始作用後，分別在 1, 10,及 20 分鐘時，加入 0.6 ml 的 0.5 M NaOH 來終止酵素反應，NaOH 除了可終止反應外，並可將產物 pNP 轉變成黃色 ，此黃色物質在 400 nm 的光照下具吸光能力 (A_{400}- absorbing）。
3. 當反應終止後，測量樣本的 A_{400}

Assay of potato acid phosphatase

馬鈴薯之酸性磷酸酶測定

0.5 M Na acetate buffer(pH 5.6)	0.12	ml
5 mM pNPP	0.24	ml
Enzyme	0.24	ml
0.5 M NaOH	0.6	ml
Sum	1.2	ml

Q.1.3. (15 points) Which enzyme concentration gave better linearity in the relationship between time and A_{400}? Circle the correct one on the Answer Sheet. Read the slope of this straight line from the graph.

在時間與 A_{400}(400nm 光源下的吸光值)的關係圖中，酶的何種濃度(1X 或 0.1X)下反應顯示較佳的線性關係？在答案卷上圈選出正確答案，並從上題作圖中計算出該直線的斜率。

Q.1.4. (5 points) Using the slope obtained in Q. 1.3, calculate the activity in the form of A_{400} change per min per 1 ml of an enzyme solution of concentration "1X". The length of the light path (L) is 1cm. Your answer should be written with your calculations and the appropriate unit in the Answer Sheet.

利用由上題中所得的斜率，計算酶的活性。方法是以其在 1 倍"1X"的酶濃度每分鐘每 ml，對 A_{400} 吸光值所造成的改變爲準，且比色管中，光所經過的路徑長度(L) 爲 1cm。在答案卷上，你所寫的答案需有單位，並要有計算過程。

Q.1.5. (5 points) Convert the absorbance change obtained in Q.1.4 to a concentration change by assuming the ε_{400} of pNP (to be 19000 $M^{-1} cm^{-1}$). Your answer should be written with your calculations and the unit per min per 1 ml of 1X enzyme solution in the Answer Sheet.

假設所得的 pNP 的吸光係數 ε_{400} 是 19000 $M^{-1} cm^{-1}$，將上題所得的吸光值變化換算成濃度變化的數值。在答案卷上，所寫的答案需以每 ml 每分鐘的 1 倍"1X"酶濃度爲單位，並需有計算過程。

Q.1.6. (5 points) Convert the concentration change obtained in Q.1.5. to a change in number of moles of pNP. Your answer should be written with your calculations in moles per min per ml of 1X enzyme solution in the Answer Sheet.

將上題所得之 pNP 濃度變化換算出 pNP 的莫耳數變化。在答案卷上，所寫的答案需以每 ml 每分鐘的 1 倍"1X"濃度的酶溶液反應下爲單位，並需有計算過程。

Q.1.7. (5 points) Calculate the total activity (in mol per min) in 4 ml of 1X enzyme solution that was initially given.

計算最初所提供的 4 ml 之 1 倍"1X"酶溶液的總活性(單位爲 mol per min；每分鐘多少莫耳)。

Task 2 (30 points)

Protein determination

蛋白質測定

Protein concentration is determined by using a standard protein such as bovine serum albumin (BSA). In Task 2, you will determine a BSA-equivalent concentration of the 1x enzyme solution by the Bradford method. The Bradford method takes advantage of an increase in absorption of Coommassie Brilliant Blue at 595 nm when it is bound to protein.

蛋白質濃度的測定常藉由使用標準蛋白質（例如：小牛血清蛋白(BSA)）來進行。在第二部分中，你將利用 Bradford 法來測定，一個與 BSA 相等濃度的 1 倍“1X”酶溶液的蛋白質的含量。此法係藉著染劑 Coommassie Brilliant Blue 與蛋白質結合時，Coommassie Brilliant Blue 染劑在 595 nm 光源下，其吸光度的增加，以進行蛋白質濃度的定量。

By diluting a concentrated BSA solution (0.4 mg protein ml^{-1}) with 3% NaCl, a 1/2-dilution series was made (0.4, 0.2, 0.1, and 0.05 mg protein ml^{-1}). The dilution series of BSA and the 0.1X enzyme solution, which was made in Task 1, were all similarly treated with dye. Optical density at 595 nm (OD_{595}) of these samples was measured and recorded in the table below.

利用 3% NaCl 溶液稀釋濃縮的 BSA 溶液(0.4 mg protein ml^{-1})，造成四種不同濃度的序列稀釋之 BSA 溶液(分別爲 0.4, 0.2, 0.1, and 0.05 mg protein ml^{-1})。此 BSA 序列稀釋溶液及其第 1 部分中所用的 0. 1 倍“0.1X”酶溶液，皆以相同的 Coommassie Brilliant Blue 染劑處理。這些樣本在 595 nm 的吸光度(OD_{595})之測量值記錄如下表中。

Table

Sample	[BSA] ($mg \cdot ml^{-1}$)	OD_{595}
	0.05	0.470
	0.1	0.543
	0.2	0.661
	0.4	0.921
0.1x enzyme solution		0.580

Optical density (OD), a measure of the extent to which a substance transmits light or the ‘absorbance’ of suspension of particles.
吸光值是指测定某種物質透光的程度，或懸浮溶液中的顆粒之吸光程度。

Q.2.1.(10 points) Plot OD_{595} against BSA concentration in the graph in the Answer Sheet and depict an approximate straight line.
將測量 OD_{595} 所得的數據結果對應於 BSA 的濃度，在答案卷所提供的方格紙上進行繪圖，並取其最接近的直線表示其趨勢。

Q.2.2.(10 points) Estimate a protein concentration of the 0.1X enzyme solution from the graph, and obtain the protein concentration of the 1X enzyme solution.
從圖中來估算 0. 1 倍“0.1X”酶溶液之蛋白質濃度，並由此換算出 1 倍“1X”酶溶液之蛋白質濃度。

Q.2.3.(10 points) Calculate the specific activity (activity per min per mg protein) of the 1x enzyme solution. Your answer should be written with your calculations and the unit per min per mg protein in the Answer Sheet.
計算 1 倍“1X”酶溶液之活性（每 mg 蛋白質每分鐘的活性），在答案卷上，你的作答必須有計算過程，及以每 mg 蛋白質每分鐘的活性爲單位（per min per mg protein）。