Lab 4. Biochemistry 385 Dr. Sugumaran's Part

Assessing the specificity of mushroom tyrosinase with combinatorially made compounds.

Recently scientists have developed a new chemical technique called combinatorial chemistry. It is an extremely valuable technique for drug discovery process. Conventionally scientists used to make one compound at a time based on certain structural predictions and see whether it could serve as a useful drug candidate. However, this process is time consuming and if it fails, they have to start all over again with a new compound. Since drug discovery takes anywhere between 10 to 15 years, it is highly desirable to cut short this time. Combinatorial chemistry allows the simultaneous synthesis as well as testing of hundreds and thousands of compounds of related structure in a single pot (or in different wells of a microtitre plate). Thus it will reduce the long time taken for developing a drug. Hence it has become very popular and most valuable tool in the drug discovery process. It is not only useful for drug discovery process, but also for a number of biochemical applications. You will perform one such application in this laboratory exercise. Today you will synthesize different substrates for the enzyme, mushroom tyrosinase using the combinatorial chemistry technique and test whether they could serve as substrates for the enzyme.

Conventional Chemistry: $\mathbf{A + B \text{----} > A\text{-}B}$

Combinatorial Chemistry: $\mathbf{A_{1\text{-}n} + B_{1\text{-}n} \text{----} > A_{1\text{-}n}B_{1\text{-}n}}$

Tyrosinase is an enzyme that belongs to the oxidoreductase class of enzymes. It catalyzes two different reactions that are shown in figure 1. These two reactions are absolutely essential for the biosynthesis of the phenolic pigment, melanin. Two kinds of melanin pigments – the red to yellow pheomelanin and the brown to black eumelanin – are produced in animals. Depending on the distribution, density and the type of melanin produced in the skin, we can get different skin colors. The red skin of Scandinavians, red coat color of fox and other animals and red feather of birds are due to pheomelanin pigment. Pheomelanin arises by the oxidative polymerization of cysteinyl dopa – an adduct of the amino acid cysteine with dopaquinone. The dark hair color, eye color and skin color observed in people of Asian and African origin is due to eumelanin. Eumelanin arises by the oxidative polymerization of 5, 6-dihydroxyindoles (Figure 2). Thus melanin is an important biopolymer. Melanin biosynthesis is initiated by the enzyme, tyrosinase. Tyrosinase oxidizes the amino acid, tyrosine, to dopa and converts the resultant dopa to dopaquinone (Figure 1). Dopaquinone undergoes nonenzymatic transformation to produce dopachrome, which is converted to 5, 6-dihydroxyindoles by other enzymes in the melanogenic pathway. These compounds are further oxidized by tyrosinase (and other related enzymes) eventually producing eumelanin pigment as the final product (Figure 2).

Tyrosinase is not only present in animals, but is also found in plants, fungi and even in some bacteria. In plants, it is responsible for the oxidative browning reactions observed during aging and cutting the vegetables. Browning of potatoes, eggplant and avocados is due to the tyrosinase action. (We add lemon juice that inhibits the enzyme tyrosinase from acting on its substrates thereby preventing the browning reaction in foods). Browning causes bitterness and

spoilage of the food material. Therefore, it is an undesired action. Similarly, browning of mushrooms (fungi) and darkening of shrimp (arthropod cuticle) are caused by the enzyme tyrosinase. Prevention of oxidative browning of shrimp alone will save the industry millions of dollars each year. In insects, it helps to make their hard exoskeleton, which protects the soft-bodies insects from their environmental enemies. Scientists are working on ways to inhibit these and other reactions specifically so that we can produce valuable products for biotechnology applications for future use.

Due to the importance of tyrosinase in melanin biosynthesis, numerous studies have been carried out on this enzyme. Inhibiting the activity of this enzyme is essential to make compounds that will serve as a) insecticides that are specific for arresting the cuticular hardening; b) skin color lightening compounds; c) sun screen lotions; and d) products that will prevent the oxidative browning of vegetable and sea food. Compounds accelerating these reactions can serve as skin tan materials. These are some of the biotechnological applications. Hence, there is a lot of research work being conducted on this enzyme.

Tyrosine shows a very broad substrate specificity. Hence it can act on a number of compounds. In this laboratory, using combinatorial chemistry technique, we will synthesize and test a number of cysteinyl catechol adducts on this enzyme.

Experimental Section:

Part A: Combinatorial Chemistry:

You are provided with a micro titre plate. First familiarize yourself with the markings on the plate. The wells are labeled. The columns are labels 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12. The rows are labeled as A, B, C, D, E, F G. and H. (12 x 8 = 96 wells total). We will use only columns 1-8 and rows A-E in this experiment.

Step 1. Add 100 µl of the catecholic compound 1 to the wells marked A, B, C, D and E under column 1.
Then take catecholic compound 2 and add to the next set of wells (2A-2E) under column 2.
Then take catecholic compound 3 and add to the next set of wells (3A-3E) under column 3.
Then take catecholic compound 4 and add to the next set of wells (4A-4E) under column 4.
Then take catecholic compound 5 and add to the next set of wells (5A-5E) under column 5.
Then take catecholic compound 6 and add to the next set of wells (6A-6E) under column 6.
Then take catecholic compound 7 and add to the next set of wells (7A-7E) under column 7.
Then take catecholic compound 8 and add to the next set of wells (8A-8E) under column 8.

The each of the column in the plate (1-8) contains the same catechols (eight compounds). The rows A-E contain eight different catechols.

(Caution work in pairs) – First read these instructions and make sure you understand. If you have doubt, talk to the instructors. Do not hurry and mess up the experiment.

We are going to oxidize all these catechols except those in rows E. So E1, E2, E3, E4, E5, E6, E7, and E8 will not receive any other chemicals. They will be unmodified compounds.

The chemicals have to be added in a particular sequence only. The sequence for each well is listed in the following table:

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
A	Catechol **1** periodate Thiol **A**	Catechol **2** periodate Thiol **A**	Catechol **3** periodate Thiol A	Catechol **4** periodate Thiol A	Catechol **5** periodate Thiol A	Catechol **6** periodate Thiol A	Catechol **7** periodate Thiol A	Catechol **8** periodate Thiol A
B	Catechol **1** periodate Thiol B	Catechol 2 periodate Thiol B	Catechol 3 periodate Thiol B	Catechol **4** periodate Thiol B	Catechol **5** periodate Thiol B	Catechol **6** periodate Thiol B	Catechol **7** periodate Thiol B	Catechol **8** periodate Thiol B
C	Catechol 1 periodate Thiol C	Catechol 2 periodate Thiol C	Catechol 3 periodate Thiol C	Catechol **4** periodate Thiol C	Catechol **5** periodate Thiol C	Catechol **6** periodate Thiol C	Catechol **7** periodate Thiol C	Catechol **8** periodate Thiol C
D	Catechol 1 periodate Thiol D	Catechol 2 periodate Thiol D	Catechol 3 periodate Thiol D	Catechol **4** periodate Thiol D	Catechol **5** periodate Thiol D	Catechol **6** periodate Thiol D	Catechol **7** periodate Thiol D	Catechol **8** periodate Thiol D
E	Catechol 1 150 µL H2O	Catechol 2 150 µL H2O	Catechol 3 150 µL H2O	Catechol **4** 150 µL H2O	Catechol **5** 150 µL H2O	Catechol **6** 150 µL H2O	Catechol **7** 150 µL H2O	Catechol **8** 150 µL H2O

Add 50 µl of sodium periodate solution to the well 1A. A yellow color develops immediately in this well. Your partner should add 100 µl of thiol A (cysteine) to this well very quickly. Do not delay. Both of you have to work coordinately and very quickly. Sodium periodate oxidizes the catechol 1 to quinone and cysteine adds on to the quinone forming cysteinyl catechol 1. The reaction is nearly quantitative and quick. As a result, you have synthesized a cysteinyl catechol 1 in this well.

Now proceed to the next well. Add 50 µl of sodium periodate to well 2A. Your partner should add 100 µl of thiol A to this well very quickly and mix the contents. You have synthesized cysteinyl catechol 2 in this well. Now proceed to the next well. We are going to do this to all the rest of the wells in the given order.

Add 50 µl of sodium periodate to well 3A. Your partner should add 100 µl of thiol A to this well very quickly and mix the contents. You have synthesized cysteinyl catechol 3 in this well. Now proceed to the next well.

Add 50 µl of sodium periodate to well 4A. Your partner should add 100 µl of thiol A to this well very quickly and mix the contents. You have synthesized cysteinyl catechol 4 in this well. Now proceed to the next well.

Add 50 µl of sodium periodate to well 5A. Your partner should add 100 µl of thiol A to this well very quickly and mix the contents. You have synthesized cysteinyl catechol 5 in this well. Now proceed to the next well.

Add 50 µl of sodium periodate to well 6A. Your partner should add 100 µl of thiol A to this well very quickly and mix the contents. You have synthesized cysteinyl catechol 6 in this well. Now proceed to the next well.

Add 50 µl of sodium periodate to well 7A. Your partner should add 100 µl of thiol A to this well very quickly and mix the contents. You have synthesized cysteinyl catechol 7 in this well. Now proceed to the next well.

Add 50 µl of sodium periodate to well 8A. Your partner should add 100 µl of thiol A to this well very quickly and mix the contents. You have synthesized cysteinyl catechol 8 in this well.

You have now completed the synthesis of eight different cysteinyl catechols in row A. We are going to synthesize the next set of thiol-catechols in the next row, namely row B. We are going to make N-acetyl cysteinyl catechols adducts in this row. We call this thiol B.

Add 50 µl of sodium periodate to well 1B. Your partner should add 100 µl of thiol B to this well very quickly and mix the contents. You have synthesized N-acetyl cysteinyl catechol 1 in this well. Now proceed to the next well.

Add 50 µl of sodium periodate to well 2B. Your partner should add 100 µl of thiol B to this well very quickly and mix the contents. You have synthesized N-acetyl cysteinyl catechol 2 in this well.

Add 50 µl of sodium periodate to well 3B. Your partner should add 100 µl of thiol B to this well very quickly and mix the contents. You have synthesized N-acetyl cysteinyl catechol 3 in this well.

Add 50 µl of sodium periodate to well 4B. Your partner should add 100 µl of thiol B to this well very quickly and mix the contents. You have synthesized N-acetyl cysteinyl catechol 4 in this well.

Add 50 µl of sodium periodate to well 5B. Your partner should add 100 µl of thiol B to this well very quickly and mix the contents. You have synthesized N-acetyl cysteinyl catechol 5 in this well.

Add 50 µl of sodium periodate to well 6B. Your partner should add 100 µl of thiol B to this well very quickly and mix the contents. You have synthesized N-acetyl cysteinyl catechol 6 in this well.

Add 50 µl of sodium periodate to well 7B. Your partner should add 100 µl of thiol B to this well very quickly and mix the contents. You have synthesized N-acetyl cysteinyl catechol 7 in this well.

Add 50 µl of sodium periodate to well 8B. Your partner should add 100 µl of thiol B to this well very quickly and mix the contents. You have synthesized N-acetyl cysteinyl catechol 8 in this well.

You have now compete the synthesis of N-acetyl cysteinyl catechol adducts in row B. Let us now proceed to make a different thiol adduct in row C. Thiol C is dithiothreitol.

Add 50 µl of sodium periodate to well 1C. Your partner should add 100 µl of thiol C to this well very quickly and mix the contents. You have synthesized dithiothreitol catechol 1 in this well. Now proceed to the next well.

Add 50 µl of sodium periodate to well 2C. Your partner should add 100 µl of thiol C to this well very quickly and mix the contents. You have synthesized dithiothreitol catechol 2 in this well.

Add 50 µl of sodium periodate to well 3C. Your partner should add 100 µl of thiol C to this well very quickly and mix the contents. You have synthesized dithiothreitol catechol 3 in this well.

Add 50 µl of sodium periodate to well 4C. Your partner should add 100 µl of thiol C to this well very quickly and mix the contents. You have synthesized dithiothreitol catechol 4 in this well.

Add 50 µl of sodium periodate to well 5C. Your partner should add 100 µl of thiol C to this well very quickly and mix the contents. You have synthesized dithiothreitol catechol 5 in this well.

Add 50 µl of sodium periodate to well 6C. Your partner should add 100 µl of thiol C to this well very quickly and mix the contents. You have synthesized dithiothreitol catechol 6 in this well.

Add 50 µl of sodium periodate to well 7C. Your partner should add 100 µl of thiol C to this well very quickly and mix the contents. You have synthesized dithiothreitol catechol 7 in this well.

Add 50 µl of sodium periodate to well 8C. Your partner should add 100 µl of thiol C to this well very quickly and mix the contents. You have synthesized dithiothreitol catechol 8 in this well.

You have now compete the synthesis of dithiothreitol catechol adducts in row C. Let us now proceed to make a different thiol adduct in row D. Thiol D is glutathione.

Add 50 µl of sodium periodate to well 1D. Your partner should add 100 µl of Thiol D to this well very quickly and mix the contents. You have synthesized glutathione catechol 1 in this well. Now proceed to the next well.

Add 50 µl of sodium periodate to well 2D. Your partner should add 100 µl of Thiol D to this well very quickly and mix the contents. You have synthesized glutathione catechol 2 in this well.

Add 50 µl of sodium periodate to well 3D. Your partner should add 100 µl of Thiol D to this well very quickly and mix the contents. You have synthesized glutathione catechol 3 in this well.

Add 50 µl of sodium periodate to well 4D. Your partner should add 100 µl of Thiol D to this well very quickly and mix the contents. You have synthesized glutathione catechol 4 in this well.

Add 50 µl of sodium periodate to well 5D. Your partner should add 100 µl of Thiol D to this well very quickly and mix the contents. You have synthesized glutathione catechol 5 in this well.

Add 50 µl of sodium periodate to well 6D. Your partner should add 100 µl of Thiol D to this well very quickly and mix the contents. You have synthesized glutathione catechol 6 in this well.

Add 50 µl of sodium periodate to well 7D. Your partner should add 100 µl of Thiol D to this well very quickly and mix the contents. You have synthesized glutathione catechol 7 in this well.

Add 50 µl of sodium periodate to well 8D. Your partner should add 100 µl of Thiol D to this well very quickly and mix the contents. You have synthesized glutathione catechol 8 in this well.

You have now compete the synthesis of glutathione catechol adducts in row D. As mentioned earlier the last row (1E- 8E) is unmodified catechols. Just add 150 ul of water to all wells in this row.

We have made 8 different quinones from 8 different catecholic compounds. We then reacted these 8 different quinones with 4 different thiols. The thiols are cysteine, N-acetyl cysteine, dithiothreitol and glutathione. This gave us 32 different compounds and along with unmodified originals, we now have a total of 40 different compounds. We are going to take an aliquot of all these compounds to another microtitre plate to assay for enzyme activity. Therefore, transfer 100 µl of aliquots from the well 2A in plate one to well 2A in Plate 2. Followed by 2B to 2B. and so on. (I hope you got the idea to transfer all 40 compounds to new plate. you are simply taking a portion and duplicating the plate for enzyme assay). We are going to use the new plate for assay and keep the original plate as a control for comparison.

Add 150 µl of 50 mM sodium phosphate buffer pH 6.5 to each of the wells in the second micro titre plate. Finally, add 20 µl of mushroom tyrosinase very quickly to all the wells in the second plate. Wait for 10 min, and record the color change in each comparison. Often it may be difficult to assess the color change. So use the original plate for comparison. Original plate will serve as the control. Any change in color intensity observed in wells between the original and the duplicate tells you about the course of enzyme reaction. If tyrosinase is acting on a substrate it will produce colored quinones in the reaction. Record the appearance of color which is an indicator of tyrosinase reaction by a + sign in the data sheet provided to you.

	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8
Cysteine (Cys) Thiol A								
N-acetylcysteine Thiol B								
Dithiothreitol Thiol C								
Glutathione Thiol D								
Unmodified catechols								

Part B: Time course of the substrate specificity studies of tyrosinase:

(Due to time constraint, we cannot test all the 40 substrates we have. We will perform the experiment for the 8 unmodified compounds only). We have eight unmodified substrates in the first microtitre plate (E1, E2, E3, E4, E5, E6, E7, and E8 in row E) . We will test these eight compounds as substrates for mushroom tyrosinase and see which work better and which do not work well.

For this part, you need the spectronic 20. Turn on the spectronic 20 machines. Set the wavelength at 420 nm. Take 4 ml of 50 mM sodium phosphate buffer, pH 6.5 in a test tube. Use it to blank the spectronic 20.

To a different test tube, add 100 µl of the compound from well 1E followed by 4 ml of 50-mM sodium phosphate buffer, pH 6.5. At time 0, add 20 µl of tyrosinase to this tube and mix the contents quickly. Immediately keep the tube in the spectronic 20 and start taking reading for every 30 sec intervals for 10 to 15 min.

Time sec	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8
0								
30								
60								
90								
120								
150								
180								
210								
240								
270								
300								
330								
360								
390								
420								
450								
480								
510								
540								
570								
600								

Plot the absorbance value for C-1 on the Y-axis against time in the X-axis.

Then repeat the experiment for the compound in well 2E. Perform the experiment similarly for 3E, 4E, 5E, 6E, 7E and 8E.

Plot all the graphs in one sheet. Use different colors for each compounds and mark them. Find out which is the best substrate for the enzyme and which is the worst substrate.