CHEM 334: Fun with Oranges!

Enzymatic ester hydrolysis is one of the first enzyme-catalyzed reactions studied in many biochemistry courses. At the University of Southampton, Professor Bugg (1, p 105) designed an undergraduate experiment for elementary bioorganic chemistry courses using the extracted esterase of an orange peel. Students performing that experiment compared regiospecificities of three different esterases. In comparison, this undergraduate experiment is very similar to Bugg’s; however, this experiment takes more of an organic approach to the workup and yield of the reaction.

In this variation of the classic orange peel esterase experiment, you will not only have the opportunity to observe an enzymatic biochemical reaction, but will have the opportunity to utilize thin layer and liquid separation chromatography and ultraviolet absorption. This is a two lab-period experiment: the first week was used to prepare the orange peel extract, set up and monitor enzymatic hydrolysis, and determine an appropriate thin layer chromatography system while the second week was used to determine which reaction mixtures proceeded and work up a reaction mixture to obtain solid products whose melting points are experimentally determined. Ideal yields for this reaction are quantitative.

Experimental Procedure

Preparation of Starting Materials

The laboratory TAs will have prepared solutions of 0.2M p-nitrophenyl acetate (pNPA, 4-nitrophenyl ethanoate) and methyl benzoate (MB) in acetone. 0.1251mL of MB is dissolved in 5mL of acetone and 181.15mg of pNPA is dissolved in 5mL of acetone. An appropriate thin layer chromatography (TLC) system was devised to be 30% ethyl acetate in hexanes for the separation of the starting materials from their alcohol and ester products.

Preparation of Orange Peel Filtrate

The laboratory TA will remove the zest of an entire orange with a citrus zester and combine it with 40mL of phosphate buffer (pH 7.0). The resulting mixture will be homogenized via blender; more buffer solution may be added to achieve such homogeneity. The mixture is vacuum filtered over a thin layer of Celite® 545. 5-10mL of buffer is used to rinse the blender and filtered material. The clear orange filtrate is collected in a beaker for use in enzyme-catalyzed hydrolysis. The extract is stable for at least 72h when stored at 2-8°C.

Hydrolysis

In 5 mL screw-topped vials, each with a micro “stir-bar,” the following four reactions are set up:

1. 6 drops pNPA solution + phosphate buffer, pH 7.0 for \( V_T=3 \)mL
2. 6 drops pNPA solution + orange peel filtrate for \( V_T=3 \)mL
3. 6 drops MB solution + phosphate buffer, pH 7.0 for \( V_T=3 \)mL
4. 6 drops MB solution + orange peel filtrate for \( V_T=3 \)mL
These four reaction mixtures are capped and allowed to stir. The reactions are monitored by TLC every 15 minutes. They are allowed to run overnight (in this case, until the next laboratory period).

**Qualitative Analysis**

For the reaction mixtures that proceeded since the first laboratory period, the products will be purified by column chromatography and a melting point obtained. A small swab of glass wool is packed into a pipette, followed by a thin layer of sea sand, silica gel, and another thin layer of sea sand to leave about a third of the pipette for solvent addition. The reaction mixtures are extracted with dichloromethane (1.5 mL/x4), dried with sodium sulfate, and evaporated to minimal volume (not solid product). The extracted, evaporated remainders were loaded onto the silica gel pipette columns and flushed thoroughly with hexanes (3-5mL). The products are eluted with an appropriate solvent system (same as thin layer chromatography system), dried in vacuo, and weighed (if an appropriate scale is available). If a mass is able to be determined, then a percent should be calculated. After weighing, the melting point of a small sample of each product is determined.

**Melting Points**

- p-nitrophenyl acetate: 78°C
- methyl benzote: -12.5°C
- p-nitrophenol: 113-114°C
- benzoic acid: 122°C

**Literature Cited**