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# Enzyme activity (10 families)

## Supplies and Reagents:

NaHPO4 buffer: pH 7, 100 mM
Substrate: Ask Jie
Cofactor: prepare the cofactor (Table 1)

Table 1. Amount of buffer, substrate, cofactors and H2O in each reaction

| enzyme family | pH buffer | substrate (final conce | cofactors (final concentration) | | | H2O |
|---|---|---|---|---|---|---|
| PAL | | | | | | |
| | 100mM | 50uM | | | | add 25 ul to 100ul |
| | 50 ul | 5 ul | | | | |
| 4CL | | | CoA | ATP | MgCl2 | |
| | 100mM | 50uM | 100uM | 2 mM | 2mM | add 16 ul to 100ul |
| | 50 ul | 5 ul | 5 ul | 2 ul | 2 ul | |
| C4H, C3H, Cald5H | | | NADPH | G6P | G6PdeH | |
| | 100mM | 50uM | 1mM | 10mM | 2ul | add 4 ul to 100ul |
| | 50 ul | 5 ul | 4 ul | 10 ul | 2 ul | |
| ComT, CoAOMT | | | MgCl2 | SAM | | |
| | 100mM | 50uM | 2mM | 250uM | | add 20 ul to 100ul |
| | 50 ul | 5 ul | 2 ul | 3 ul | | |
| CAD | | | NADPH | G6P | G6PdeH | |
| | 100mM | 50uM | 1mM | 10mM | 2ul | add 20 ul to 100ul |
| | 50 ul | 5 ul | 4 ul | 10 ul | 2 ul | |
| CCR | | | NADPH | G6P | G6PdeH | |
| | 100mM | 50uM | 1mM | 10mM | 2ul | add 4 ul to 100ul |
| | 50 ul | 5 ul | 4 ul | 10 ul | 2 ul | |
| HCT | | | shikimic acid | | | |
| | 100mM | 50uM | 500 uM | | | add 24 ul to 100ul |
| | 50 ul | 5 ul | 1 ul | | | |

## Important Notes:

Before HPLC, use the centrifuge for all the tubes in 20,000Xg for 30 min**.**

## Protocol:

### 1. Enzyme reaction

1. For each enzyme family, add the buffer, substrate, cofactors and $H_2O$ to 6 tubes (Table 1). 3 Tubes will used as control, and 3 tubes will used as reaction.
2. 3 Tubes will used as background, and add the buffer (50 ul) and H2O (30 ul) to each tube.
3. 3 Tubes will used as reference for #22, and add the same cofactors as CAD.
4. 3 Tubes will used as reference for #24, and add the same cofactors as CAD.
5. Incubate all the tubes in 30 C.

6. Start the reaction by adding 20 ul xylem protein to reaction tubes. For control, background, and reference tubes, add 20 ul boiled xylem protein.
7. Stop the incubation of all tubes (except the CAD reaction tubes and reference tubes) at 30 min by adding 3M TCA (5 ul).
8. Stop the incubation of CAD and reference tubes at 30 min by heating the tubes in 99 C for 5 min.
9. Use the centrifuge for all the tubes in 20,000Xg for 30 min.
10. Transfer the supernatural to HPLC insert.

**2. Preparation of the equipment**

1. Check the column, in this analysis C18 column will be used.
2. Turn on the five sections of the equipment, click on Instrument online to set up for the analysis.
3. Check the level of the bottle before starting any sequence. Fill if necessary and reset the volume of the bottles.
4. A standard method for the enzyme activity named Enzyme activity 1 on the HPLC machine should be used.
5. Set up sequence and run.

**3. Data analysis**

1. Get the peak area by using Rt and wave length in Table 2.
2. Use the template to get the relative activity and specific activity of each enzyme.

Table 2. Rt and Wave length for each compound

| emzyme family | substrate | Rt(min) | wave length (nm) |
|---|---|---|---|
| PAL | Phenylalanine | | |
| C4H | Cinnamic acid | 9.442 | 280 |
| 4CL | p-Coumaric acid | 5.352 | 310 |
| 4CL | Caffeic acid | 3.817 | 325 |
| HCT | p-Coumaroyl-CoA | 4.503 | 325 |
| C3H | p-Coumaroyl shikimic acid | 4.903 | 310 |
| CCoAOMT | Caffeoyl-CoA | 3.81 | 325 |
| CCR | Feruloyl-CoA | 4.709 | 325 |
| CAld5H | Coniferaldehyde | 8.385 | 325 |
| COMT | 5-Hydroxyconiferaldehyde | 6.427 | 325 |
| CAD/SAD | Coniferaldehyde | 8.385 | 325 |
| CAD/SAD | Sinapaldehyde | 8.31 | 325 |
| | p-Couamaryl alcohol | 5.956 | 260 |
| | Coniferyl alcohol | 6.548 | 260 |
| | Sinapyl alcohol | 6.437 | 280 |