COEI-1-CINEST Measurement of cinnamoyl esterase activity in enzymatic preparations

Two different methods are proposed to measure the cinnamyl esterase activity since we have no principal precursor, para-coumaroyltartric acid; the first method uses the chlorogenase activity of *Aspergillus niger* i.e. the hydrolysis of chlorogenic acid (caffeoylquinic); this requires the implementation of conventional enzymatic measuring apparatus.

The second method relates to the hydrolysis of ethyl cinnamate, the content of which is measured by gas chromatography.

Both methods were compared, their give similar results.

General specifications

Unless otherwise stipulated, the specifications must comply with the resolution OIV/OENO 365/2009 concerning the general specifications for enzymatic preparations included in the International Oenological Codex.

1. Origin

These enzymatic activities are often present in preparations like pectolytic enzymes by directed fermentations of *Aspergillus sp.*

2. Scope of application

This activity is responsible for the production of volatile phenols which impacts negatively the sensory properties of wines, especially white wines. On the other hand, only limited studies have demonstrated that this activity seems to have a positive effect in stabilising the colour of red wines.

3. Method A. Chlorogenate hydrolase or chlorogenase (EC. 3.1.1.42 - CAS no. 74082-59-0)

3.1. Principle

Cinnamyl esterase degrades chlorogenic acid releasing caffeic acid. The reduction in measured absorbance at 350 nm linked to the disappearance of this substrate can be used to quantify the cinnamyl esterase activity.

An enzymatic unit is defined as being the quantity of enzyme enabling a drop in the absorbance of 1 unit at pH 6.5 and 30°C.

3.2. Apparatus

3.2.1. water bath at 30°C

3.2.2. water bath at 100°C

3.2.3. litre graduated flask

3.2.4. 125-mL Erlenmeyer flask

3.2.5. 100-mL graduated flask

3.2.6. 1000-mL graduated flask

3.2.7. Chronometer

3.2.8. 100- µL precision syringe

3.2.9. 1000- µL precision syringe

3.2.10. 5000- µL precision syringe

3.2.11. graduated 5-mL straight pipette

3.2.12. pH-meter

3.2.13. spectrophotometer

3.2.14. 15 mL glass screw-top test tubes

3.2.15. metal rack for 15-mL test tubes

3.2.16. cuvets with a 1-cm optical path length, for single use, for spectrophotometer, for measurement in the UV spectrum

3.2.17. stirrer of the Vortex type

3.3. Products

3.3.1. methanol (Analytical Reagent Rank – CH_3OH - PM = 32.04 g/mole)

3.3.2. sodium dihydrogenophosphate ($NaH_2PO_4.2H_2O$ 99% pure - PM = 156.01 g/mole)

3.3.3. sodium hydroxide (NaOH 99% pure - PM = 40 g/mole)

3.3.4. chlorogenic acid (95% pure - PM = 354.30 g/mole)

3.3.5. distilled water

3.3.6. commercial enzymatic preparation for analysis

3.4. Solutions

3.4.1. Methanol at 80% (v/v)

Introduce 100 mL of methanol (3.3.1) into a 125-mL Erlenmeyer flask (3.2.4) to which 25 mL of distilled water (3.3.5) have been added.

3.4.2. Sodium hydroxide solution at 9M:

Introduce 360g of sodium hydroxide (3.3.3) into a 1000-mL graduated flask (3.2.6) and make up with distilled water (3.3.5).

3.4.3. Phosphate buffer 0.1M (pH 6.5)

Introduce 31.5 g of sodium dihydrogenophosphate (3.3.2) into a 2-litres graduated flask (3.2.3) to which 1.8 litres distilled water (3.3.5) have been added.

Adjust the pH to 6.5 using the sodium hydroxide solution (3.4.2) and a pH-meter (3.2.11). Then adjust the volume with 2 litres with distilled water (3.3.5).

3.4.4. Chlorogenic acid solution at 0.06% (p/v)

Dissolve 0.06 g of chlorogenic acid (3.3.4) in a 100-mL graduated flask (3.2.5) to which the phosphate buffer (3.4.3) has been added up to the gauge line.

3.5. Preparation of the sample

It is important to homogenise the enzymatic preparation before sampling, by upturning the container for example. The enzymatic solution and the blanks will have to be prepared extemporaneously.

3.5.1. Enzymatic solution at 10 g/L

- Place 1g of commercial preparation (3.3.6) in a 100-mL graduated flask (3.2.5), make up with the phosphate buffer (3.4.3), and stir (3.2.17) in order to obtain a homogeneous mixture.

    2. White denatured by heating

- Place 10 mL of the enzymatic solution at 10 g/L (3.5.1) in a 15-mL test tube (3.2.14) and immerse the tube for 5 minutes in the water bath at 100°C (3.2.2).

3.6. Procedure

3.6.1. Enzymatic reaction: The test tubes are produced at least in duplicate.

In 4 x 15-mL test tubes (3.2.14) numbered from 1 to 4, placed in a rack (3.2.15)

Introduce 100 µL of the enzymatic solution at 10 g/L (3.5.1), using the precision syringe (3.2.8),

500 µL of the chlorogenic acid solution (3.4.4), start the chronometer (3.2.7).

After shaking (3.2.17), the test tubes are placed in the water bath at 30°C (3.2.1)

- for 120 min. for test tube no.1
- for 240 min. for test tube no.2
- for 330 min. for test tube no.3

- for 400 min. for test tube no.4

The reaction is stopped by adding 5 mL of methanol at 80% (3.4.1) using a straight pipette (3.2.11) in each of the numbered tube 1 to 4, immediately after they have been removed from the water bath at 30°C. The tubes are then shaken.

3.6.2. Proportioning of the released substances (caffeic acid)

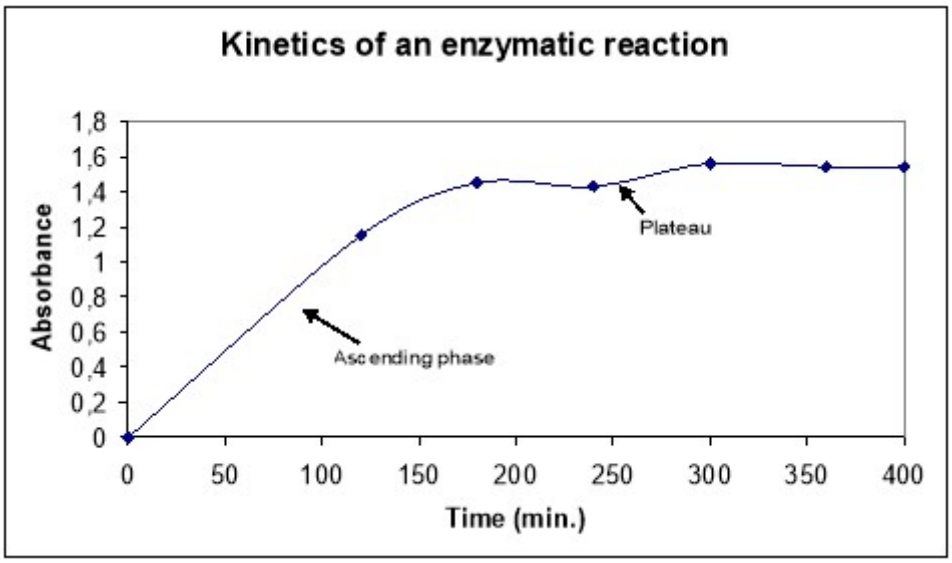
The reactional medium (3.6.1) is placed in a cuvet with a 1-cm optical path length (3.2.16). Immediately measure the absorbance at 350 Nm, using a spectrophotometer (3.2.13). The measurement is to be compared with a blank of methanol 80% pure (3.4.1).

3.7. Calculations

3.7.1. Determining the kinetics

In general, calculating the enzymatic activity can only be done when the substrate and the enzyme are not in limiting quantities. This therefore refers to the ascending phase of the kinetic representation: the enzymatic activity is linear in time. Otherwise, the activity would be underestimated (Figure 1)

Figure 1: Kinetics of an enzymatic reaction



The kinetics is determined over 400 minutes. The activity concerned is measured at T=120 min T=180 min, T=240 min, T=300 min T=360 min T=400 min.

After determining the kinetics of the enzymatic reaction, plot the curve for the variation in absorbance in relation to reaction time. The absorbance corresponds to the difference between the absorbance at time T of the enzymatic preparation and that of the corresponding blank.

Then calculate the equation (1) of the straight regression line, taking into account only the points of the ascending phase (see figure 1).

3.7.2. Calculation of the enzymatic activity

The cinnamyl esterase activity is calculated based on the reduction in absorbance per hour since this activity is very weak in the preparations. The calculation formula is as follows

$$Activity\ in\ U/g = 1000 \times ((DO0 - DOT)/T)/(V \times C)$$

- DO0: Value of the absorbance of the blank
- DOT: Value of the absorbance at time T (hour)
- V: quantity of enzymatic solution introduced (µL), in this case 100 µL
- C: concentration of the enzymatic solution (g/L), in this case 10 g/L

4. Method B. Hydrolysis of ethyl cinnamate

4.1. Principle

Cinnamyl esterase hydrolyses ethyl cinnamate. The reduction in this ester measured by gas chromatography can be used to quantify the cinnamyl esterase activity.

4.2. Apparatus

4.2.1. Gas phase chromatograph with a flame ionisation detector or mass spectrometry equipped with a capillary tube of the Carbowax 20 M type 50 m x 0.2 mm x 0.2 µm phase thickness

4.2.2. Magnetic stirrer and stirrer bars

4.2.3. Laboratory glassware (5-mL precision pipettes, conical flasks, 50-mL and 100-mL graduated flasks, 10-mL, 60-mL, 150-mL laboratory glass bottles etc.)

4.2.4. Pasteur pipettes

4.2.5. 200- µL, 50- µL and 10- µL precision syringes

4.2.6. Drying oven at 25°C

4.2.7. Precision balance to within 0.1 mg/L

4.2.8. pH-meter

4.3. Products

4.3.1. Methanol (Analytical Reagent Rank – CH3OH - PM = 32.04 g/mole)

4.3.2. Citric acid 99% pure

4.3.3. Sodium hydroxide (NacOH 99% pure - PM = 40 g/mole)

4.3.4. Ethyl cinnamate (99% pure - PM = 176 g/mole)

4.3.5. Distilled or permuted water

4.3.6. Commercial enzymatic preparation for analysis

4.3.7. Pure ethanol 99% vol.

4.3.8. Diethylic ether 99% pure.

4.3.9. Pure Dodecanol

4.4. **Solutions**

4.4.1. Ethanol at 12% (v/v)

Introduce 12 mL of ethanol (4.3.7) into a 100-mL graduated flask (4.2.3) make up to volume with distilled water (3.3.5).

4.4.2. Sodium hydroxide solution 4 M

Introduce 16 g of pure sodium hydroxide into a 100-mL graduated flask; make up with distilled water; stir until dissolution.

4.4.3. Citrate buffer at pH 6.5

Introduce 0.05 g of citric acid (4.3.2) into a 150 mL bottle (4.2.3), add 100 mL of ethanol to 12% vol. (4.4.1) dissolve using a magnetic stirrer. Place under magnetic stirring in the presence of the electrode of the pH-meter (4.2.8) bring to pH 6.5 byadding the sodium hydroxide 4 M drop by drop (4.4.2).

4.4.4. Stock solution of ethyl cinnamate at 500 mg/L

Using a precision syringe (4.2.5) place 50 µL of ethyl cinnamate (4.3.4) in a 100-mL graduated flask containing a little pure ethanol (4.3.7) make up to the gauge line with pure ethanol (4.3.7); homogenise

4.4.5. Ethyl cinnamate solution at 25 mg/L in the citrate buffer

In a 100-mL graduated flask, place 5 mL of stock solution of ethyl cinnamate at 500 mg/L (4.4.4) measured with a precision pipette (4.2.3); make up to 100 mL with the citrate buffer at pH 6.5 vol. (4.4.3). Homogenise.

Note: a more concentrated ethyl cinnamate solution must not be prepared because the ester is liable to be partially insoluble.

4.4.6. Dodecanol solution at 0.5 g/L(internal standard)

Using a precision syringe (4.2.5) place 50 µL of pure dodecanol (4.3..9) in a 100-mL graduated flask containing a little pure ethanol (4.3.7); make up the gauge line with pure ethanol (4.3.7); homogenise.

4.5. Preparation of the sample

It is important to homogenise the enzymatic preparation before sampling, by upturning the container for example.

_4.6. Procedure

4.6.1. Enzymatic reaction: In a 60-mL laboratory flask, place 50 mL of ethyl cinnamate solution at 25 mg/L (4.4.5) add approximately 100 mg of the commercial enzymatic preparation to be analysed (4.3.6) weighed with precision (4.2.7), i.e. weight P.

After stirring (4.2.2), the bottle is plugged and left on the laboratory bench or if possible in a drying oven at 25°C (4.2.6)

4.6.2. A sample of 200 µL is taken with a precision syringe (4.2.5) after 3 hours, 24 hours, 72 hours.

4.6.3. The reaction is stopped by adding the sample (4.6.2) of 200 µL in a 10-mL flask containing 0.5 mL of methanol (4.3.1) and 1 mL of ether (4.3.8)

4.6.4. Addition of the internal standard

In the preparation (4.6.3), using a precision syringe (4.2.5) add 50 µL of dodecanol to 500 mg/L (4.4.6); homogenise.

4.6.5. Blank

Proceed as in 4.6.3 and 4.6.4 without adding the 200 µL of the sample from the enzymatic reaction (4.6.2)

4.6.6. Reference solution

Proceed as in 4.6.3 and 4.6.4 by placing in the bottle (4.2.3) 200 µL of ethyl cinnamate solution at 25 mg/L (4.4.5) instead of the sample of enzymatic reaction (4.6.2)

4.6.7. Chromatography

4.6.7.1. Inject 2 µL of the blank (4.6.5) into the chromatograph to locate the internal standard. Start the temperature programmer and the data acquisition.

4.6.7.2. Inject 2 µL of reference solution to locate the ethyl cinnamate (ec) and the internal standard (is); measure their respective surface areas Sec0 Sis0

4.6.7.3. Under the same conditions as 4.6.7.2 inject the samples (4.6.4) after 3 hours, after 24 hours and after 72 hours, i.e. the respective surface areas of residual ethyl cinnamate and internal standard S3 and Sis3; S24 Sis24, S72 Sis72.

Determine the quantity of residual ethyl cinnamate for each sample; for example for 72 hours.

$$EC72 = \frac{25 \times Sei0}{SCE0} \times \frac{SCE\ 72}{Sei72}$$

$$EC\ consumed\ in\ 72\ hours = 25 - EC\ 72$$

Cinnamyl esterase activity in mg of hydrolysed ethyl cinnamate per hour and g of

enzymatic preparation

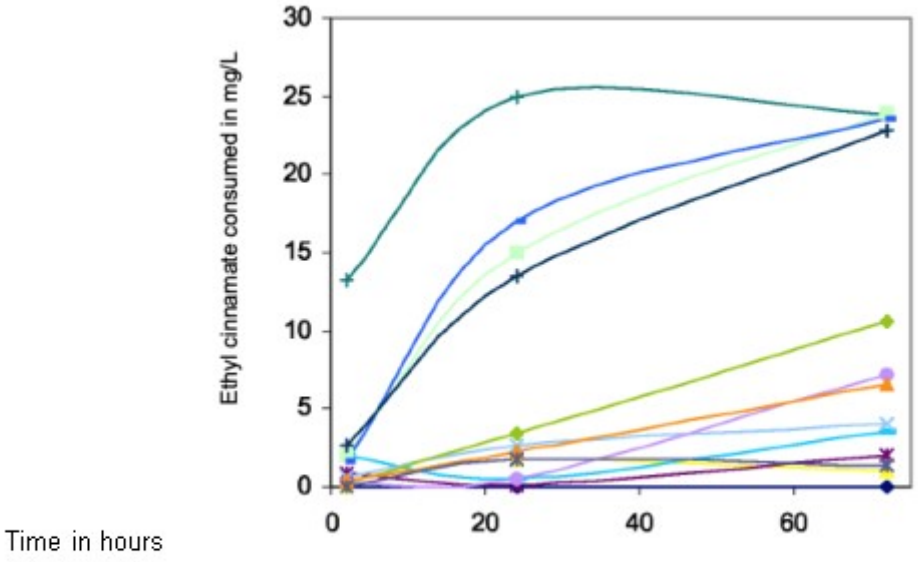
EC activity in EC mg/g enzyme/hour =

$$\frac{25 \times EC\ 72 \times 1000}{P \times 25 \times 72}$$

- P = weight of enzyme added in the preparation (6.1) in mg/L.

4.7. Comments: The method has been freely adapted from Barbe (1995).

The reaction taking place at pH 6.5 is much more complete than with the pH in the wine where it is approximately 10 times slower; therefore, if after 72 Hours, only a few mg of ethyl cinnamate have been degraded, the EC activity in the wine can be considered negligible.



Examples of cinnamoyl esterase activities measured at pH 6.5, of commercial enzymatic preparations.

5. Bibliography

- Barbe CH, 1995: On the contaminating esterase activities of pectolytic preparations. PhD Thesis, Univer