RESOLUTION OIV-OENO 625-2021

COMPARATIVE EVALUATION OF PROTEASE ACTIVITY (ASPERGILLOPEPSIN I) IN ENZYME PREPARATIONS

THE GENERAL ASSEMBLY,

IN VIEW OF the Article 2, paragraph 2 b) ii of the Agreement of 3rd April 2001 establishing the International Organisation of Vine and Wine,

CONSIDERING the works of the "Specification of Oenological Products" Expert group,

CONSIDERING the resolutions [OIV-OENO 541A-2021] Use of Aspergillopepsin I to remove haze-forming proteins in grape must and [OIV-OENO 541B-2021] Use of Aspergillopepsin I to remove haze-forming proteins in wine,

CONSIDERING this determination is only suitable to compare proteolytic activity in enzyme preparations,

DECIDES on the proposal of Commission II "Oenology" to add the following monograph in chapter 1 of the International Oenological Codex:

Comparative evaluation of protease activity (Aspergillopepsin I) in enzyme preparations

1. Origin

Enzyme preparations that have an Aspergillopepsin I activity are formed by controlled fermentation of Aspergillus spp., in particular of Aspergillus niger.

This enzyme is usually referred to as Aspergillopepsin I or Aspergillus acid protease (EC 3.4.23.18). Proteases are usually present as an enzyme complex. Unless otherwise stated, the specifications of resolution OIV-OENO 365-2009 must comply with "general specifications of enzyme preparations" set out in the International Oenological Code.

Reference is made to paragraph 5 "Source of enzyme and fermentation environment" of the general monography on Enzymatic preparations.

2. Scope/ Applications

Reference is made to the International Code of Oenological Practices, OIV-OENO

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541A-2021 and OIV-OENO 541B-2021.

Enzymatic preparations containing protease activities (Aspergillopepsin I) are able to degrade the native must or wine protein under specific conditions of heat treatment. These proteins are causing great difficulties during must and wine clarification and stabilization steps. Such Proteases are therefore specifically used for the stabilization of protein rich must and wine.

In order to verify that the treatment has led to the removal of proteases (Aspergillopepsin I) and to the reduction of the native level of proteins, the proteins can be assayed in finished wines using the SDS-PAGE method described in the Appendix I of this monograph.

3. Principle

This procedure is only for the determination of proteolytic activity in enzyme preparations, expressed in spectrophotometric acid protease units (SAPU), of preparations derived from, e.g., Aspergillus niger, and Aspergillus oryzae. The test is based on a 30-min enzymatic hydrolysis of a Hammarsten Casein Substrate at pH 3.0 and 37 °C. Unhydrolyzed substrate is precipitated with trichloroacetic acid and removed by filtration. The quantity of solubilized casein in the filtrate is determined spectrophotometrically (reference: Food Chemical Codex).

4. Reagents and solutions

4.1. Casein: Use Hammarsten-grade casein, (CAS 9000-71-9, e.g. Merck article number 102242)

4.2. Glycine-Hydrochloric Acid Buffer (0.05 M): Dissolve 3.75 g of glycine in about 800 mL of water. Add 1 M hydrochloric acid until the solution is pH 3.0, determined with a pH meter. Quantitatively transfer the solution to a 1000-mL volumetric flask, dilute to volume with water, and mix.

4.3. TCA Solution: Dissolve 18.0 g of trichloroacetic acid and 11.45 g of anhydrous sodium acetate in about 800 mL of water and add 21.0 mL of glacial acetic acid. Quantitatively transfer the solution to a 1000-mL volumetric flask, dilute to volume with water, and mix.

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4.4. Substrate Solution: Pipet 8 mL of 1 M hydrochloric acid into about 500 mL of water and disperse 7.0 g (moisture-free basis) of Casein (4.1) into this solution, using continuous agitation. Heat for 30 min in a boiling water bath, stirring occasionally, and cool to room temperature. Dissolve 3.75 g of glycine in the solution and adjust to pH 3.0 with 0.1 M hydrochloric acid, using a pH meter. Quantitatively transfer the solution to a 1000-mL volumetric flask, dilute to volume with water, and mix.

5. Sample preparation

Weigh the enzyme preparation, quantitatively transfer it to a glass mortar, and triturate with Glycine-Hydrochloric Acid Buffer (4.2).

Quantitatively transfer the mixture to an appropriately sized volumetric flask, dilute to volume with Glycine-Hydrochloric Acid Buffer (4.2), and mix.

The solution of the sample enzyme preparation must be prepared so that 2 mL of the final dilution gives a corrected absorbance of enzyme incubation filtrate at 275 nm (A, as defined in the Procedure) between 0.200 and 0.500.

6. Procedure

- Pipet 10.0 mL of Substrate Solution (4.4) into each of a series of 25 x 150 mm test tubes, allowing at least two tubes for each sample, one for each enzyme blank, and one for a substrate blank.
- Stopper the tubes, and equilibrate them for 15 min in a water bath maintained at 37°C ± 0.1°C.
- At zero time, start the stopwatch, and rapidly pipet 2.0 mL of the Sample Preparation into the equilibrated substrate.
- Mix by swirling and replace the tubes in the water bath. (Note: The tubes must be stoppered during incubation).
- Add 2 mL of Glycine-Hydrochloric Acid Buffer (instead of the Sample Preparation) to the substrate blank.
- After exactly 30 min, add 10 mL of TCA Solution (4.3) to each enzyme incubation and to the substrate blank to stop the reaction. (Caution: Do not use mouth suction for the TCA Solution).

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- In the following order, prepare an enzyme blank containing 10 mL of Substrate Solution, 10 mL of TCA Solution, and 2 mL of the Sample Preparation.
- Heat all tubes in the water bath for 30 min, allowing the precipitated protein to coagulate completely.
- At the end of the second heating period, cool the tubes in an ice bath for 5 min, and filter through Whatman No. 42 filter paper, or equivalent. The filtrates must be perfectly clear.
- Determine the absorbance of each filtrate in a 1-cm cell at 275 nm with a suitable spectrophotometer, against the substrate blank. Correct each absorbance by subtracting the absorbance of the respective enzyme blank.

6.1. Standard Curve

- Transfer 181.2 mg of L-tyrosine, chromatographic-grade or equivalent (CAS 60-18-4, e.g. Merck article number 108371), previously dried to constant weight, to a 1,000-mL volumetric flask.
- Dissolve in 60 mL of 0.1 M hydrochloric acid.
- When completely dissolved, dilute the solution to volume with water, and mix thoroughly. This solution contains 1.00 µmol of tyrosine in 1.0 mL.
- Prepare dilutions from this stock solution to contain 0.10, 0.20, 0.30, 0.40, and 0.50 µmol per mL.
- Determine the absorbance of each dilution in 1-cm cell at 275 nm, against a water blank.
- Prepare a plot of absorbance versus µmol of tyrosine per mL. A straight line must be obtained.
- Determine the slope and intercept for use in the Calculation below. A value close to 1.38 should be obtained for the slope. The slope and intercept may be calculated by the least squares method as follows:

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$$Slope = \left[n\sum(MA) - \sum(M)\sum(A)\right] / \left[n\sum(M^2) - (\sum M)^2\right]$$

$$Intercept = \left[\sum(A)\sum(M^2) - \sum(M)\sum(MA)\right] / \left[n\sum(M^2) - (\sum M^2\right]$$

in which n is the number of points on the standard curve, M is the µmoL of tyrosine per ml for each point on the standard curve, and A is the absorbance of the sample.

6.2. Calculation

One spectrophotometric acid protease unit is that activity that will liberate 1 µmoL of tyrosine per min under the conditions specified. The activity is expressed as follows:

SAPU/g = (A – I) x 22 / (S x 30 x W)

in which

- **A** is the corrected absorbance of the enzyme incubation filtrate;
- **I** is the intercept of the Standard Curve;
- **22** is the final volume of the incubation mixture, in mL;
- **S** is the slope of Standard Curve;
- **30** is the incubation time, in min; and
- **W** is the weight, in g, of the enzyme sample contained in the 2.0-mL aliquot of sample preparation added to the incubation mixture in the Procedure.

Appendix I: SDS-PAGE protein assay

1. Principle

This test is based on a modified Bradford method (Marchal et al., 1997; Marchal et al., 1996) combined with SDS-PAGE electrophoresis.

The quantification of proteins is realized with a Bradford test using an ultrafiltration at 3kDa to reduce the interferences due to ethanol and phenolic compounds (Marchal et al., 1996), and SDS-PAGE (Sodium Dodecyl Sulfate - PolyAcrylamide Gel) electrophoresis to separate the proteins according to their molecular weight

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(Laemmli, 1970).

2. Protocol

The samples (wines before treatment, wines with Aspergillopepsin I just added, wines after treatment) are ultrafiltrated with centrifuge filters 3 kDa (for example: Amicon® Ultra-4, Merck Millipore, Irlande) at 4500 g during 20 minutes at 18 °C, and the ultrafiltrate is collected.

400 µL ultrapure water are added to 400 µL of sample (wine or ultrafiltrate) and 200 µL Bradford reagent (Bio-Rad, USA) in a semi micro-cuvette (path length 10mm).

The solution is mixed twice, and the absorbance is measured at 595 nm after 30 minutes, compared to ultrapure water.

To obtain the absorbance of proteins (A_P), the absorbance of the ultrafiltrate (A_UF) has to be deduced from the absorbance of wine (A_W):

$$A_P = A_V - A_{UF}$$

A standard curve with 5 concentrations (from 0 to 20 mg/L) is made with BSA (Bovin Serum Albumin) with 10-minutes reaction. The total protein content is calculated in mg/L eq. BSA, with the average value of 3 different measures.

Polyacrylamide gels are used, at 4% for stacking and 13% for resolving (composition in Table 1).

Samples are mixed with Laemmli buffer 4X (3 volumes of samples + 1 volume of buffer; Bio-Rad, USA) and analyzed by SDS-PAGE. Markers from 10 to 250 kDa are used as standards: Precision Plus Protein TM Unstained Standards, Bio-Rad, USA. The analyses are made by triplicate.

Table 1. Composition of resolving and stacking gels (for 4 gels).

Composition	**Resolving gel (13%)**	**Stacking gel (4%)**
Ultrapure water	6.2 mL	4.88 mL
Bis-Acrylamide (30%)	8.6 mL	1.04 mL
Buffer Tris-HCl 1,5M pH 8.8	5.0 mL	-

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Buffer Tris-HCl 0,5M pH 6,8	-	2.0 mL
Sodium dodecyl sulfate (SDS) 10%	0.2 mL	80 µL
Ammonium Persulfate (APS) 10%	100 µL	40 µL
Tetramethylethylenediamine (TEMED)	20 µL	8 µL

Gels are run on a vertical electrophoresis apparatus (for example: Mini-PROTEAN III; Bio-Rad, USA) at room temperature and stained with Coomasie blue R250.

After migration, gels are stained with silver nitrate at room temperature according Rabilloud (1994): see Table 2.

Table 2. Protocol for silver staining SDS-PAGE gels.

Step	**Solution: final concentration**	**Time**
Fixing	Ethanol 99%: 30% (v/v) Acetic acid: 10% (v/v)	Over night
Sensitization	Ethanol 99%: 20% (v/v) Potassium acetate: 0.5M Potassium tetrathionate: 3 g/L Glutaraldehyde 50%: 1% (v/v)	2 h 30 min (in the dark)
Washing	Ultrapure water	3 x 20 min
Staining	Silver nitrate: 2 g/L Formaldehyde 37%: 0.7 mL/L	30 min
Washing	Ultrapure water	15 sec
Development	Potassium carbonate: 30 g/L Formaldehyde 37%: 0.5 mL/L Sodium thiosulfate. $5H_2O$ 2.48 g/L: 3.75 mL/L	5 min
Stop	Tris: 50 g/L Acetic acid: 25 mL/L	5 min

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3. Results

The molecular weight of chitinases and TLP (Thaumatin Like Proteins) is below 15 kDa and the proteases' one is close to 40 kDa. A visual analysis of the gels allows an initial observation of the residual proteins.

Precise results are obtained after digitalization of the SDS-PAGE gels and analysis with a specific software.

4. References

1. Marchal R., Seguin V. et Maujean A. Quantification of interferences in the direct measurement of proteins in wines from the Champagne region using the Bradford method. American Journal of Enology and Viticulture, 1997, 48, 303-309.
2. Marchal R., Bouquelet S. et Maujean A. Purification and partial biochemical characterization of glycoproteins in a Champenois Chardonnay wine. Journal of Agricultural and Food Chemistry, 1996, 44, 1716-1722.

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