Determination of β-Glucanase activity in Rovabio by viscosimetry

1. Document history

Not relevant

2. Warnings

Refer to each product safety data sheet.
Wear suitable safety glasses and gloves.
Work under fume cupboard.

3. Scope

This method is applicable to all samples, either in a liquid or in a powder form.

4. Normative references

Not applicable.

5. Definitions

Not applicable.

6. Principle and reactions

In this method are described the different operations to be performed to determine β-glucanase activity using a viscosimetric method.

6.1. Principle

This analysis is specific to endo-1,3(4)-β-glucanase activity. The assay is based on the enzymatic hydrolysis of a barley β-glucan standard solution. The enzymatic activity is determined from the relative viscosity reduction.

6.2. Definition of the unit

Each unit of endo-1,3(4)-β-glucanase activity is equivalent to the quantity of enzyme that hydrolyzes the substrate, thus reducing the solution viscosity, in order to change the relative fluidity by one unit per minute without dimension, according to the analysis conditions.

The standard β-glucanase activity determination is performed at pH 5.5 and 30 °C. However, an activity determination can also be performed at different pH and temperatures

in order to characterize the protein. In this case, the buffer pH or even the buffer type may be modified, as well as the enzymatic reaction temperature.

7. Reagents and materials

All reagents must be of analytical grade. Except when specifically indicated, the water used is chromatographic grade water. The water used in the water bath can be tap or distilled water.

The reagents used for the β-glucanase activity determination using the viscosimetric method are presented in annex 1. In every case, but the β-glucan, the identity as well as the purity of the reagents are more important than the supplier criteria.

7.1. Barley β-glucan at 1 % (w/V)

To prepare 100 mL of substrate, weigh exactly about 1.000 g to the nearest 0.001 g of β-glucan. Place 70 mL of water in a beaker, add a magnetic stirring bar and place on a heating magnetic stirrer. Stir vigorously and start heating. When the water is hot (but not boiling), add the β-glucan in avoiding the formation of lumps. When the liquid starts to boil, stop the heating, cover up the beaker with a watch glass and continue the stirring for 10 more minutes in order to completely dissolve the substrate. Then cool down to room temperature (20 - 25°C) (at this step, the beaker can be placed onto a cold magnetic stirrer). Transfer the solution in a 100 mL volumetric flask. Add 10 mL of pH 5.5 1M sodium acetate buffer solution. Rinse the beaker with water and transfer into the volumetric flask. Fill up the flask to volume using water.

Leave the solution to settle in a refrigerator for 18 hours prior use.

Keep the substrate in a refrigerator for a maximum of 2 weeks.

7.2. 1M sodium acetate

Dissolve approximately 82.0 g weighed to the nearest 0.2 g of anhydrous sodium acetate in 500 mL of water. Stir vigorously using a magnetic stirrer. Transfer in a 1L volumetric flask, rinse the beaker with water and transfer into the volumetric flask. Fill up the flask to volume using water.

Transfer into a bottle. Keep at room temperature for a maximum of 2 months.

7.3. 1M acetic acid

Weigh exactly approximately 60.1 g to the nearest 0.2 g of glacial acetic acid in a 100 mL beaker. Place a 500 mL beaker containing 400 mL of water onto a magnetic stirrer. Stir and pour slowly the previously weighed acetic acid. Transfer in a 1L volumetric flask. Rinse the beaker with water and transfer into the volumetric flask. Fill up the flask to volume using water.

Transfer into a bottle. Keep at room temperature for a maximum of 2 months.

7.4. pH 5.5 1M sodium acetate buffer solution

Using a calibrated pH meter, add some 1M acetic acid solution to the sodium acetate solution until pH 5.5 is reached.

Transfer into a bottle. Keep at room temperature for a maximum of 2 months.

7.5. pH 5.5 0.01M sodium acetate buffer solution

Pipette 10 mL of 1M sodium acetate buffer solution in a 1 L volumetric flask. Fill up to volume using water.

8. Apparatus

- Usual glassware and laboratory equipment;
- Disposable sterile tubes with stoppers;
- P5000 and P1000 GILSON type pipettes and electronic HandyStep pipette;
- Stopwatch;
- Heating magnetic stirrer + magnetic stirring bars;
- Calibrated pH meter and analytical balance;
- Water bath capable of 30.0 °C (± 0.1 °C);
- Vortex;
- Microviscosimeter (e.g. AMVn type ANTON PAAR) + associated software;
- Ultrasonic bath.

The whole of the equipment has to be used in a clean environment in order to avoid any eventual contamination problems. The environment should be isolated from any concentrated enzyme sample and should be cleaned regularly. It is better practice to use pipettes specifically dedicated to this type of analysis.

References of the equipment used to date are listed in annex 2 as an example. Any change of supplier reference should be validated through a comparative study. The measurement equipment used should have similar characteristics than the equipment described in this method.

9. Sampling

Not applicable.

10. Procedure

10.1. Sample preparation

Every enzymatic preparation will be diluted in pH 5.5 0.01M sodium acetate buffer solution. Try to minimize the dilution errors (no small enzyme volumes in large water volumes). In every case, it is better practice to weigh out the enzyme to be analyzed, even for a liquid enzyme. The sample density, measured at 20°C, will be used to calculate the activity in U / mL of the product.

The enzyme is extracted at room temperature under magnetic stirring, for 10 min. for AP formulations, for 30 min. for AP T. FLEX formulations and manually for LC formulations.

Classical assay portions are:

- approximately 0.600 g weighed to the nearest 0.001 g for AP formulations, in 250 mL pH 5.5 0.01M sodium acetate buffer solution;
- approximately 0.900 g weighed to the nearest 0.001 g for LC formulations, in 100 mL pH 5.5 0.01M sodium acetate buffer solution;
- approximately 0.900 g weighed to the nearest 0.001 g for LC2 formulations, in 200 mL pH 5.5 0.01M sodium acetate buffer solution.

If necessary, dilute the extract to reach an enzyme concentration within the linearity range of the method.

The enzyme has to be diluted in order to be within the 5 - 15 minutes test measuring interval:

$$\frac{\Delta\,(\text{viscosity decrease})}{\Delta\,\text{time}} = -105 \pm 10\ m\,\text{sec}/\text{min}$$

This corresponds to a 950 - 1150 msec decrease during the (approximate) 10 minutes measurement time.

10.2. Sample assay

Set the microviscosimeter with an appropriate capillary and a ball (see annex 3).

Before to start any enzymatic analysis, the capillary and the ball have to be calibrated against the buffer (buffer blank) and against the substrate (substrate blank). These calibrations must be performed each day an analysis is performed.

It is necessary to use clean equipment. The capillary has to be cleaned with water then dried with a flux of nitrogen between each analysis.
At the end of the day, clean the capillary and the ball using water, then ethanol then acetone and dry it with a flux of nitrogen.

10.2.1. Calibration against the diluted buffer solution - Buffer blank determination

Place 5 mL of pH 5.5 0.01M sodium acetate buffer solution in a disposable sterile tube. Sonicate for 5 seconds. Place at 30 °C for 5 minutes.
Fill up the capillary (containing a calibrated ball) with 30 °C pH 5.5 0.01M sodium acetate buffer solution. Make sure that no air bubble remains in the capillary, then place it into the microviscosimeter.
The microviscosimeter will perform 60 measurements. The drop time average for the *40 last measurements* for the buffer is labelled Tw (value equal to about 12 s).

10.2.2. Calibration against the substrate - Substrate blank determination

Place 3 mL of water and 1 mL of 1 % barley β-glucan solution in a disposable sterile tube. Sonicate for 5 seconds then place at 30 °C for (at least) 5 minutes.
Add 1 mL of pH 5.5 0.01M sodium acetate buffer solution to the substrate at 30 °C. Mix using a vortex then fill up the capillary containing a calibrated ball with the diluted

barley β-glucan solution (make sure that no air bubble is present in the capillary). Insert the capillary into the microviscosimeter.
The microviscosimeter will perform 30 measurements. The drop time average for the *20 last measurements* for the substrate is labelled Ts (value equal to about 24 s).

If the obtained value is too low or if the drop time decrease is lower than -10 msec / min, discard the substrate and prepare a new substrate solution.

10.2.3. Enzymatic activity determination for the sample

Place 3 mL of water and 1 mL of 1 % barley β-glucan solution in a disposable sterile tube. Sonicate for 5 seconds then place the tube in a thermostated water bath at 30 °C and leave to equilibrate for (at least) 5 minutes.
Add 1 mL of enzymatic preparation solution to the substrate at 30 °C. Start the stopwatch. Mix using a vortex then fill up the capillary containing a calibrated ball with the mixture (make sure that no air bubble is present in the capillary). Insert the capillary into the viscosimeter after exactly 1 minute.
The viscosimeter will perform 30 measurements. At the end of the analysis, remove the capillary, clean it, and then dry it with a flux of nitrogen.

11. Calculation

11.1. Determination of the enzymatic activity

Fr	=	relative fluidity
Tw	=	average drop time for the buffer (msec)
Ts	=	average drop time for the substrate (msec)
Tt	=	drop time during the assay at a given t time (msec)
t	=	assay time (minutes)
$T_{1/2}$	=	assay time (t) plus half (½) of Tt (minutes)
DF	=	dilution factor
5	=	total test volume (mL)
V	=	enzyme solution volume in the sample (1 mL)
Correction Factor	=	substrate factor (specific to each substrate batch)

① For the ***20 last measurements***, calculate Fr for each Tt

$$Fr = \frac{T_s - T_w}{T_t - T_w}$$

② Plot Fr = f ($T_{1/2}$)
③ Determine the linear regression slope
④ From it, determine the raw output activity: the obtained slope is proportional to the enzymatic activity present in the sample.

$$Raw\ output\ activity\ (U/g) = slope \times DF \times \frac{5}{V}$$

⑤ Determine the final activity

$$Activity\ (U/g) = Activity \times Correction\ Factor$$

Note: When changing substrate, results could be different. Thus, apply a correction factor to get identical results as those obtained when using the former substrate.

11.2. Determination example

Calibrated ball	=	1.5 mm (steel 1.4125)
Enzyme dilution	=	0.5027 g in 100 mL then 1 mL in 100 mL then 0.72 mL in 25 mL (D = 690715)
Tw	=	12048 msec
Ts	=	24480 msec
Slope Fr	=	0.0137
Activity	=	0.0126 x 690715 x (5/1)
	=	**43515 U / g**

11.3. Results interpretation

For the two series of analysis, calculate the difference between the two results:

$$Difference = \frac{Value_{max.} - Value_{min.}}{Value_{min.}} \times 100$$

If the difference is lower than or equal to 5 %, calculate the average of the two values to obtain the result.
If the difference is higher than 5 %, a third determination will have to be performed.

11.4. Non compliant results

Substrate blank: the viscosity reduction maximum (dropping time) that should be obtained during the calibration is - 10 msec / min. Lower values could indicate a contamination of the substrate or the capillary by an enzyme. Clean thoroughly the capillary and repeat the calibration. If the problem remains, prepare a new substrate solution.

12. Precision

N/A

13. Special cases

Not applicable.

14. Annexes

Annex 1: Reagents list.
Annex 2: Equipment list.

Annex 3: AMVn type microviscosimeter settings.

15. Bibliography

N/A

Annex 1

List of reagents used and associated references

Reagent	Supplier	Product reference	Molecular weight (g / mol)
β-glucan	MEGAZYME LTD	P-BGBM	-
Anhydrous sodium acetate	PROLABO	27 653 292	82.03
Acetic acid	PROLABO	20 104 298	60.05
Acetone		CAS 97-64-1	

Annex 2

Equipment used and associated references

Consumables

- P1000 cones — GILSON — Ref.: D1000ST
- P5000 cones — GILSON — Ref.: D5000
- 25 mL Combitips plus — EPPENDORF
- 12.5 mL Combitips plus — EPPENDORF

Equipment

- Stopwatch (precision count-up / count-down) — VWR — Ref.: 609-0016
- METTLER AE200 analytical balance, 0.1 mg precision
- Vortex SCIENTIFIC INDUSTRIES, Vortex Genie 2 model
- METTLER TOLEDO MP230 pH meter, 0.1 pH unit precision
- GILSON pipettes P1000
  P5000
- HANDYSTEP electronic pipette
- GRANT Y14 water bath, 0.1 °C precision
- ANTON PAAR (AMVn) microviscosimeter + associated software

Annex 3

Measurement equipment used and microviscosimeter settings

Apparatus type	AMVn type Anton Paar microviscosimeter	Supplier: ANTON PAAR
Capillary	1.6 mm internal diameter capillary	Supplier: ANTON PAAR
Ball	1.5 mm diameter steel ball	Supplier: ANTON PAAR
Measurement angle	75°	
Measurement temperature	30 °C	
Number of measurements for the water blank	60	The 20 first values are not taken into account in the calculations
Expected value for the water blank	About 12 seconds	
Number of measurements for the substrate blank	30	The 10 first values are not taken into account in the calculations
Expected value for the substrate blank	About 24 seconds	
Number of measurements for the sample assay	30	The 10 first values are not taken into account in the calculations