Endo(1→3)(1→4)-β-D-Glucanase (Malt β-Glucanase)

Background

Malt β-glucanase [endo 1,3(4)-β-D-glucanase; EC 3.2.1.73] activity is an indicator of malt quality and has a functional role in the depolymerisation of barley β-glucan [(1→3)(1→4)-β-D-glucan] during seed germination and during the mashing process of beer production (particularly if mashing-in temperatures below 50°C are employed).[1] Even limited depolymerization of barley β-glucans by this enzyme will significantly reduce or alleviate the filtration problems they cause and it will also reduce the tendency for these polymers to precipitate from solution in wort and beer.

A number of methods for the measurement of malt β-glucanase have been proposed and these include reducing sugar assays, viscometric assays and procedures which take advantage of the interaction of barley β-glucan with either Calcofluor or Congo Red e.g. the radial diffusion method. However, none of these methods has gained acceptance by official brewing bodies. The IRVU viscometric method was routinely used and recommended by the Institute of Brewing (U.K.) until recently when it was dropped due to its lack of interlaboratory reproducibility.[2]

Polysaccharide substrates with covalently attached dye molecules are commonly used for the assay of a number of endo-hydrolases. In the method currently described, such a substrate was prepared from highly purified barley β-glucan by carboxymethylating with chloroacetic acid (to increase solubility) and dying with Remazolbrilliant Blue R.[3,4,5]

Scope

The determination of endo-β-glucanase content of malt using chemically modified, dye-labelled, barley glucan as substrate.

Field of Application

The method can be applied to all malts. With slight adjustment of the pH of the extraction buffer, it can also be used to measure the activity of cellulases [endo-(1→4)-β-D-glucanases; EC 3.2.1.4] and bacterial β-glucanase [lichenase; EC 3.2.1.73] on barley β-glucan.

Principle

Ground malt is extracted using a buffer solution at pH 4.6 and the resulting solution is centrifuged.

The malt extract is then allowed to react with Azo-Barley glucan substrate under defined conditions. The dyed substrate is depolymerised by β-glucanase to give fragments which are soluble in the presence of a precipitant solution.

On centrifugation of the precipitant-treated reaction mixture, the absorbance (at 590nm) of the supernatant solution is related to the level of β-glucanase in the extracted malt.

The method is calibrated using a previously standardised sample of malt flour.

Reagents

1. Azo-Barley glucan substrate (10g/litre) in sodium azide solution (0.2g/litre). Store at 0 - 5°C between use. Prior to dispensing the substrate for the estimation, warm the vial to 40°C and vigorously shake the contents for 5 to 10 seconds. This is necessary to redisperse any precipitate that may form on standing or storage.

2. Concentrated buffer solution. Sodium acetate (0.80 mol/l) plus sodium phosphate (0.80 mol/l) stabilised with 0.2g/l of sodium azide.

Dilute 25.0mL of the concentrated buffer solution to 500mL with distilled water before use. This diluted extractant buffer solution contains sodium acetate (0.04 mol/l) and sodium phosphate (0.04 mol/l) which gives a buffer of pH 4.6.

Store at 0 - 5°C between use. Shelf life at this temperature is approximately 1 month.

[Alternatively, the extractant buffer solution, sodium acetate (0.04 mol/l) plus sodium phosphate (0.04 mol/l) at pH 4.6 can be prepared as follows:

Dissolve 5.44g sodium acetate ($CH_3.COONa.3H_2O$) and 6.24g of sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$) in 900mL distilled water. Add 0.2g sodium azide, adjust to pH 4.6 by the addition of hydrochloric acid (1 mol/L) and adjust the volume to 1L].

3. Precipitant solution. Dissolve 40.0g of sodium acetate trihydrate and 4.0g zinc acetate in 150mL of distilled water. Adjust the pH to 5.0 with concentrated hydrochloric acid and make the volume up to 200.0mL. To this solution add 800.0mL of methyl cellosolve and mix well.

Note. Methyl cellosolve is poisonous.

4. Malt flour of known β-glucanase activity in U/Kg.

Apparatus

Centrifuge tubes (12mL capacity); bench centrifuge; positive displacement pipettor (e.g. Eppendorf Multipipette with a 12.5mL tip) to dispense viscous substrate solution; pipettor (e.g. Finnpipette or Gilson) to dispense malt extract; adjustable volume dispensors (0 - 5.0mL for precipitant solution; 0 - 10.0mL for acetate buffer); Balance, accuracy of $\pm$ 0.05g; spectrophotometer and 1cm cells; vortex mixer; water bath at 30.0 $\pm$ 0.2^{o}C; stop clock; Buhler-Miag Universal Laboratory Disc Mill (type DLFU).

Procedure

Sample

1. Finely grind 20g of the malt sample in a Buhler-Miag disc mill at a setting of 0.2mm.

2. Weigh accurately 0.5g of the finely ground malt into a centrifuge tube of 12mL capacity.

3. Weigh accurately 0.5g of the standard malt flour into another centrifuge tube.

4. To each tube add 8.0mL of the diluted extractant buffer solution and mix the contents thoroughly using a vortex mixer.

5. Allow the extraction to proceed for 15 minutes at room temperature (less than 30^{o}C) with mixing every 5 minutes.

6. Centrifuge the tubes and contents at 1,000$\acute{g}$ for 10 minutes. Remove supernatant solution (containing extracted β-glucanase enzymes) with a Pasteur pipette into a clean tube.

. Dispense (with a positive displacement dispensor) 0.5mL aliquots for the Azo-barley glucan substrate solution (prewarmed to approximately 40°C) into centrifuge tubes (12mL capacity) and place in a water bath for 5 minutes to attemperate to 30.0°C.

. Place the centrifuge tubes containing the malt extracts from 6 (above) in the water bath at 30°C for 5 minutes.

. Pipette 0.5mL of the attemperated malt extract solution into a centrifuge tube containing the attemperated Azo-barley glucan substrate, mix the contents vigorously (vortex mixer) and maintain a temperature of 30.0°C for exactly 10 minutes, as measured from the time of addition. Start subsequent samples at 30 second intervals.

10. At the end of the 10 minute period add 3.0mL of the precipitant solution and stir the mixture vigorougly (vortex mixer).

11. Allow the centrifuge tubes to stand for 5 minutes at room temperature.

12. Centrifuge the tube and contents at 1,000g for 10 minutes. Separate the supernatant solutions immediately.

13. Using a 1cm cell, measure the absorbance of the supernatant of each sample and the reaction blank at 590nm against distilled water.

Blank

1. Add 3.0mL of the precipitant solution to 0.5mL of the Azo-barley glucan substrate and mix thoroughly.

2. Add 0.5mL of malt extract, mix thoroughly and keep at 30°C for 10 minutes and centrifuge as described for the samples.

3. A single reaction blank only is usually sufficient for each batch of malt samples analysed since the blank absorbance value does not vary significantly from sample to sample.

4. Absorbance values of reaction blanks should be approximately 0.05 when measured against distilled water in a 1cm cell at 590nm.

Expression of Results

Calculation

1. Calculate the β-glucanase content of the malt sample in U/Kg using the following formula:

$$Y = MX + C$$

where Y = Malt β-glucanase activity in U/Kg (U is International Units of activity, where one unit is the amount of enzyme which cleaves one micromole of glycosidic linkages per minute under defined conditions of temperature and pH)

M = Constant

X = Absorbance of sample solution minus absorbance of blank solution at 590nm (1cm cell).

C = Constant

2. Note that values of M and C vary slightly between batches of Azo-barley glucan and are provided with each sample of substrate.

3. Calculate a similar value for the standardised sample of malt flour.

4. Calculate the corrected β-glucanase content using the following formula:

$$\text{Corrected } \beta\text{-glucanase (U/Kg)} = Y \times \frac{A}{B}$$

where Y = Malt β-glucanase activity (in U/Kg) as calculated using the formula in 1 above.
A = Activity value for the control malt flour.
B = Activity value for the control malt flour as calculated using the formula in 1 above.

Notes

1. Estimate the malt β-glucanase content within one hour of the extraction.

2. Take care not to contaminate the Azo-barley glucan substrate solution with malt extract. Blank absorbance values measured against distilled water should be approximately 0.05 in 1cm cells at 590nm. If values are significantly higher than this value, or if they are increasing with time, discard the substrate.

3. If absorbance values for a particular extract are more than twice the value obtained for the standardised malt flour, then dilute the extract with an appropriate volume of the extractant buffer and repeat the determination.

   Adjust the calculation to take into account the dilution factor.

Accuracy of the Assay

The Cereal Chemistry Division of the Royal Australian Chemical Institute carried out an extensive collaborative testing of this method in 15 Australian and 3 European laboratories. Six samples were analysed and of the 108 assays (18 x 6) carried out, only one showed up as a statistical outlier. The coefficient of variation for each sample was calculated. The mean figure was 5.5% with a range of 4.5 to 6.1%.[6]

References

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