STARCH DAMAGE

ASSAY PROTOCOL

K-SDAM

08/23

(200 Assays per Kit)

AACC Method 76-31.01

ICC Method No. 164

Play Training Video

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INTRODUCTION:

The milling of wheat causes physical damage to a proportion of the starch granules of flour. The level of starch damage directly affects water absorption and dough mixing properties of the flour and is thus of technological significance. Furthermore, damaged granules rapidly hydrate and are hydrolysed by α- and β-amylases, yielding fermentable sugars. In the latter stages of traditional long-fermentation procedures, when the natural sugars present in the flour have been fermented by yeast, maltose produced by amylolysis of damaged starch provides a further supply of substrate. In the absence of this additional source of fermentable sugars, inadequate gassing occurs, and the resultant bread has a low loaf volume and a heavy texture.

Methods used to measure starch damage can be broadly grouped into four classes: extraction procedures ("Blue Value"), dye-staining procedures, NIR procedures and enzyme digestion procedures. Of these, the enzyme digestion procedures are generally preferred. These procedures employ α-amylase, β-amylase or combinations of these enzymes. Cereal and fungal α-amylase preparations have been employed as crude malt extracts, or crude fermentation broths. The degree of hydrolysis has traditionally been measured using non- specific reducing-sugar methods such as ferricyanide titration, or reaction with dinitrosalicylic acid (DNS).

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PRINCIPLE:

In the current procedure, damaged starch granules are hydrated and hydrolysed to maltosaccharides plus α-limit dextrins by carefully controlled treatment with purified fungal α-amylase. The fungal α-amylase treatment is designed to give near complete solubilisation of damaged granules with a minimum breakdown of undamaged granules. This reaction is terminated with the addition of dilute sulphuric acid, and aliquots are treated with excess levels of purified amyloglucosidase to give complete degradation of starch-derived dextrins to glucose. The glucose is specifically measured with a high purity glucose oxidase/peroxidase reagent mixture. Determined values are presented as starch (damaged) as a percentage of flour weight on an "as is" basis.

ACCURACY:

Standard errors of ± 3% are achieved routinely within our laboratory. A summary of the interlaboratory evaluation results used for accreditation of AACC Method 76-31.01 and AACC Method 76-31.01 appears on page 7 of this assay protocol.

KITS:

Kits suitable for performing 200 assays of starch damage are available from Neogen. The kits contain the full assay method plus:

Bottle 1: **Fungal α-Amylase** (10 mL, 1,000 U/mL on Ceralpha reagent* at pH 5.4 and 40°C). Ammonium sulphate suspension.
Store at 4°C. See individual label for expiry date.

*Full assay procedure is available at "www.megazyme.com" - Product Code: **K-CERA**.

Bottle 2: **Amyloglucosidase** (4 mL, 200 U/mL on soluble starch at pH 4.5 and 40°C). Ammonium sulphate suspension.
Store at 4°C. See individual label for expiry date.

Bottle 3: **GOPOD Reagent Buffer** (50 mL, pH 7.4), *p*-hydroxybenzoic acid and sodium azide (0.09% w/v).
Store at 4°C. See individual label for expiry date.

Bottle 4: **GOPOD Reagent Enzymes.** Glucose oxidase plus peroxidase and 4-aminoantipyrine. Freeze- dried powder.
Store below -10°C. See individual label for expiry date.

Bottle 5: D-Glucose standard solution (5 mL, 1.5 mg/mL) in 0.2% (w/v) benzoic acid.
Store sealed at room temperature. See individual label for expiry date.

Bottle 6: Wheat flour standard. Level of starch damage shown on vial label.
Store sealed at room temperature. See individual label for expiry date.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Ensure the contents of **bottle 1** are completely resuspended by swirling before removing aliquots. Dilute an aliquot (1.0 mL) to 20 mL with **buffer A** (see page 3 for preparation). Divide into appropriately sized aliquots and store in polypropylene tubes below -10 °C between use and keep on ice during use. This is **solution 1** (α-Amylase, 50 U/mL).
Stable for > 3 years below -10°C.

2. Dilute an aliquot (1.0 mL) of the contents of **bottle 2** to 10 mL with **buffer A** (see page 3 for preparation). This is **solution 2** (Amyloglucosidase 20 U/mL).
Stable for ≥ 3 years below -10°C.

3. Dilute the contents of the **GOPOD Reagent Buffer** bottle to 1 L with distilled water (this is **Solution 3**). Use immediately.

NOTE:

1. On storage, salt crystals may form in the concentrated buffer. These must be completely dissolved when this buffer is diluted to 1 L with distilled water.
2. This buffer contains 0.09% (w/v) sodium azide. This is a poisonous chemical and should be treated accordingly.

4. Dissolve the contents of the **GOPOD Reagent Enzymes** bottle in 20 mL of **solution 3** and quantitatively transfer this to the bottle containing the remainder of **solution 3**.
Cover this bottle with aluminium foil to protect the enclosed reagent from light. **This is Glucose Determination Reagent (GOPOD Reagent)**.
Stable for ≥ 1 month at 4°C or ≥ 12 months below -10°C.

If this reagent is to be stored in the frozen state, preferably it should be divided into aliquots. Do not freeze/thaw more than once.

When the reagent is freshly prepared, it may be light yellow or light pink in colour. Upon storage at 4°C the **GOPOD reagent** may develop a stronger pink colour The absorbance of this solution should be less than 0.05 when read against distilled water.

5 & 6. Use bottles 5 & 6 as supplied.

REAGENTS (NOT SUPPLIED):

1. Buffer A (100 mM Sodium acetate buffer, pH 5.0 with 5 mM calcium chloride.
- Add 5.7 mL of glacial acetic acid (1.05 g/mL) to 900 mL of distilled water. Adjust the pH to 5.0 by careful addition of 2 M (8 g/100 mL) sodium hydroxide solution. Approx. 60 mL is required.
- Add 0.74 g of calcium chloride dihydrate and dissolve. Adjust the volume to 1 L and store the buffer at 4°C. This is **Buffer A.**

2. Dilute sulphuric acid (0.2% v/v).
Add 2.0 mL of concentrated sulphuric acid to 998 mL of distilled water.
Store at room temperature.

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (round bottomed, 16 x 100 mm, 12 mL capacity).
2. Micro-pipettors, 100 µL (e.g. Gilson Pipetman® or Rainin EDP-2® motorised dispenser).
3. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® (to dispense 0.1 mL aliquots of **solution 2** (Amyloglucosidase 20 U/mL)).

- with 12.5 mL Combitip® (to dispense 1 mL aliquots of **solution 1** (α-Amylase, 50 U/mL)).
- with 50 mL Combitip® (to dispense 8.0 mL aliquots of dilute sulphuric acid and 4.0 mL aliquots of **GOPOD Reagent**).

4. Bench centrifuge (required speed 3,000 rpm, i.e. approx.1,000 *g*) OR Whatman No. 1 filter paper/ Whatman GF/A glass fibre filter papers. (See Page 5, Pt. 1).
5. Analytical balance.
6. Spectrophotometer set at 510 nm.
7. Vortex mixer.
8. Thermostated water bath set at 40°C.
9. Stop clock.

CONTROLS AND PRECAUTIONS:

1. The time of incubation of the flour samples with **solution 1** (α-Amylase, 50 U/mL) must be carefully controlled (i.e. 10 min).
   - The time of incubation with **solution 2** (Amyloglucosidase 20 U/mL) is not critical but should be at least 10 min.
   - The time of incubation with **GOPOD reagent** is not critical but should be at least 20 min. The colour complex formed is stable at 40°C for at least 2 h.
2. In step 4 of the Assay Procedure, it is essential to stir the tube and contents immediately and vigorously on addition of **solution 1** (α-Amylase, 50 U/mL) to prevent clumping of the flour.
3. In step 5 of the Assay Procedure, it is essential that the sample aliquots be delivered to the bottom of the test tubes. This ensures that all the sample will mix with the amyloglucosidase which is added in excess quantities.
4. With each set of determinations, reagent blanks and glucose standards of 150 µg should be included, in duplicate.
   - The **reagent blank** consists of 0.2 mL of **Buffer A** + 4.0 mL **GOPOD reagent**.
   - The **glucose standard** consists of 0.1 mL of **Buffer A** + 0.1 mL **bottle 5** (glucose standard, 150 µg/0.1 mL) + 4.0 mL **GOPOD reagent**.
5. With each set of determinations at least one control wheat flour should also be included.
6. The enzyme preparation solutions should not be cross-contaminated.

NOTE:

1. If a bench centrifuge is not available, the suspensions, after the addition of the dilute sulphuric acid, can be filtered through Whatman No. 1 filter papers, or Whatman GF/A glass fibre filter papers.
2. Round-bottomed glass test tubes are recommended. Mixing problems and clumping of the sample occur with conical test tubes. With polycarbonate test tubes, temperature equilibration problems may be experienced.
3. In step 4 of the Assay Procedure, some of the sample clings to the side of the test tube. This does not affect the accuracy of the assay.
4. **GOPOD reagent** should be stored on ice when in use.
5. The use of a multi-dispensing pipettor significantly reduces the time requirements when several samples are being analysed concurrently. These dispensers also improve the reproducibility and convenience of the assay.
6. The absorbances at 510 nm of some samples with very high starch damage levels may exceed that of the glucose standard. If this occurs, an aliquot of the solution obtained from step 6 of the Assay Procedure should be suitably diluted further (usually 2-fold) with 0.2% sulphuric acid and the assay repeated from step 7 of the procedure. This dilution should be accounted for in the final calculation (page 6) or in the 'dilution' column in the accompanying Megacalc available on the product webpage.

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ASSAY PROCEDURE:

1. Accurately weigh 100 ± 10 mg of wheat flour or milled gelatinised starch sample into a thick-walled glass centrifuge tube (16 x 120 mm; 12 mL capacity).

2. Pre-equilibrate the tubes plus contents at 40°C for approx. 5 min.

3. Pre-equilibrate **solution 1** (α-Amylase, 50 U/mL) at 40°C for approx. 5 min in a small glass beaker.

4. Add 1.0 mL of pre-equilibrated **solution 1** (α-Amylase, 50 U/mL) to each tube, stir the tube on a vortex mixer for approx. 5 sec and incubate at 40°C for exactly 10 min (from time of addition of the enzyme).

5. Add 8.0 mL of dilute sulphuric acid solution (0.2% v/v) to each tube after exactly 10 min from the time of addition of **solution 1** and stir the tube vigorously for approx. 5 sec. This inactivates the enzyme and thus terminates the reaction.

6. Centrifuge the tubes at 3,000 rpm (1,000 *g*) for 5 min or filter the slurry through Whatman No. 1 (9 cm) filter paper.

7. Carefully and accurately transfer 0.1 mL aliquots of the supernatant solution (or filtrate) to the bottom of two test tubes.

8. Add 0.1 mL of **solution 2** (Amyloglucosidase 20 U/mL) to each tube, stir the tubes on a vortex mixer and incubate them at 40°C for 10 min.
9. Add 4.0 mL of **GOPOD reagent** solution to each tube (including glucose standards and reagent blank tubes) and incubate the tubes at 40°C for 20 min.
10. Measure the absorbance of all solutions at 510 nm against a reagent blank.

CALCULATION OF STARCH DAMAGE LEVEL:

NOTE: These calculations can be simplified by using the Megazyme ***Mega-Calc***™ downloadable from where the product appears on the Megazyme website (www.megazyme.com).

$$\text{Starch Damage, \%} = \Delta A \times F \times 90 \times \frac{1}{1000} \times \frac{100}{W} \times \frac{162}{180}$$

$$= \Delta A \times \frac{F}{W} \times 8.1$$

where:

ΔA = Absorbance (reaction) read against the reagent blank.

$F = \dfrac{150\ (\mu g\ \text{of glucose})}{\text{absorbance of } 150\ \mu g\ \text{of glucose}}$ (conversion from absorbance to µg)

90 = volume correction (0.1 mL taken from 9.0 mL).

$\dfrac{1}{1000}$ = Conversion from micrograms to milligrams.

$\dfrac{100}{W}$ = Factor to express Starch Damage as a percentage of flour weight.

W = The weight in milligrams ("as is" basis) of the flour weight.

$\dfrac{162}{180}$ = Adjustment from free glucose to anhydro glucose (as occurs in n starch).

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CORRELATIONS WITH STANDARD METHODS:

1. SD% (AACC 76-30A) = 1.4 x SD% (Megazyme) - 0.09 (r = 0.99; n = 21)
2. SD% (Wooster) = 1.2 x SD% (Megazyme) + 0.5 (r = 0.99; n = 21)
3. SD% (Farrand) = 5.2 x SD% (Megazyme) - 10.3 (r = 0.98; n = 26)
4. SD% (Barnes) = 1.5 x SD% (Megazyme) + 0.44 (r = 0.96; n = 45)

INTERLABORATORY EVALUATION OF MEGAZYME METHOD:

Interlaboratory evaluation of the method involving 24 laboratories and 10 samples was performed in a split level (Youden Pairs) experimental design in accordance with AOAC INTERNATIONAL guidelines. Results were statistically analysed according to Australian Standard 2850-1986 (based on ISO 5725). Within laboratory coefficient of variation (%) values ranged from 2.9 to 6.8 and between laboratory coefficient of variation (%) values ranged from 5.0 to 10.3% for the different pairs. Highest coefficient of variation values were obtained for samples with the lowest degrees of starch damage.

Based on these results, the procedure has been adopted by the Cereal Chemistry Division of the Royal Australian Chemical Institute and the American Association of Cereal Chemists (AACC Method 76-31.01). The method has also been evaluated and accepted by the International Association for Cereal Science and Technology (ICC Method No. 164).

REFERENCES:

1. Gibson, T. S., Al Qalla, H. & McCleary, B. V. (1991). An improved enzymatic method for the measurement of starch damage in wheat flour. *J. Cereal Sci.*, **15**, 15-27.

2. Gibson, T. S., Kaldor, C. J. & McCleary, B. V. (1993). Collaborative evaluation of an enzymic starch damage assay kit. *Cereal Chem.*, **70**, 47-51.

3. Evers, A. D. & Stevens, D. J. (1985). Starch Damage, "*Advances in Cereal Science and Technology*", **Vol. VII**. (Pomeranz, Y. Ed.), American Association of Cereal Chemists Inc., St. Paul, Minnesota, pp. 321-349.

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