Automatic Spectrophotometric Determination of Amyloglucosidase Activity Using *p*-Nitrophenyl-α-D-glucopyranoside and a Flow Injection Analyser

Kaj André Holm
Enzyme Microbiological laboratory, NOVO Research Institute, DK 2880 Bagsvaerd, Denmark

An automated flow injection analysis (FIA) method has been developed, using as a chromophore *p*-nitrophenyl-α-D-glucopyranoside, which is hydrolysed to glucose and *p*-nitrophenol by amyloglucosidase. The *p*-nitrophenol is determined spectrophotometrically at 400 nm as a measure of the enzyme activity. The general conditions of the analysis have been optimised. The analytical method is flexibile, as all methods for the analysis of enzymes using soluble *p*-nitrophenyl derivatives as chromophores may in principle be carried out with the FIA method described. The final result is a simple, automated method with high precision, sensitivity and speed.

Keywords: *Amyloglucosidase activity determination; spectrophotometry; p-nitrophenyl-α-D-glucopyranoside; automated flow injection analysis*

A general flow injection analysis (FIA) method was required for the determination of the activity of hydrolytic enzymes, using as chromophores various soluble *p*-nitrophenyl derivatives, which under the influence of the given hydrolytic enzyme split off the *p*-nitrophenol (yellow at alkaline pH) under standardised conditions. The amyloglucosidase hydrolysis of *p*-nitrophenyl-α-D-glucopyranoside is used here as a model system.

The amyloglucosidase (*exo*-α-1,4-glucan glucohydrolase, E.C. 3.2.1.3) is a glycoprotein which contains carbohydrate residues that are glycosidically linked through D-mannose to the hydroxy groups of serine and threonine in the polypeptide chain of the enzyme.[1] The enzyme is produced by submerged fermentation of a strain of *Aspergillus niger*. The enzyme catalyses the stepwise hydrolysis of the α-1,4-linkages and also, at a slower rate, the α-1,6-bonds in liquefied starch, dextrins and oligosaccharides, by releasing single glucose units from the non-reducing end of the molecule.

Most methods for the determination of the activity of amyloglucosidase described in the literature are based on the following principle. The maltose is hydrolysed by the enzyme to form glucose, which can then be enzymatically quantified.[2,3] The work reported here uses a simpler analytical principle in which the *p*-nitrophenol released by the hydrolysis can be determined spectrophotometrically after a pH change as follows.[4–7] At pH 4.3, amyloglucosidase hydrolyses the colourless *p*-nitrophenyl-α-D-glucopyranoside (pNPG) forming glucose and *p*-nitrophenol (pNP). By adjusting the pH to basic values, the yellow colour resulting from *p*-nitrophenol can be determined spectrophotometrically at 400 nm as a measure of the enzyme activity.

Experimental

This method gives a relative measurement compared with a manually standardised amyloglucosidase standard.[6] One NOVO amyloglucosidase unit (1 AGU) is the amount of enzyme which under given standardised conditions hydrolyses 1 μM of maltose per minute at 25 °C and pH 4.3.

The following standard conditions were used for the FIA method: chromophore, *p*-nitrophenyl-α-D-glucopyranoside, 2 g l^{-1}; buffer, acetate, 0.1 M; incubation pH, 4.3; incubation temperature, 50 °C; incubation time, 20 s; colour reaction time 3 s; total analysis time 24 s.

The flow injection analysis equipment used consisted of a sampler (Tecator 5007), a pump unit (Tecator 5020), a print unit (Alfacom Sprinter 40), a recorder (Servogor 120), a spectrophotometer (Shimadzu UV 120–02) and a cuvette (Helma QS 178–720).

Reagents

All chemicals were of analytical-reagent grade.

Acetate buffer solution, pH 4.3, 0.1 M. The solution is prepared by dissolving 4.44 g of sodium acetate trihydrate and 3.75 ml of glacial acetic acid in 1 l of de-mineralised water.

p-*Nitrophenyl-α-D-glucopyranoside solution*, 2 g l^{-1} (6.64 mmol l^{-1}). The reagent is prepared by dissolving 0.200 g of *p*-nitrophenyl-α-D-glucopyranoside in 100 ml of acetate buffer (pH 4.30, 0.1 M). The reagent is prepared freshly every day and must be protected against light.

Sodium carbonate solution, 0.1 M, *pH* 11.6. This is prepared by dissolving 10.6 g of sodium carbonate in 1 l of de-mineralised water.

Amyloglucosidase standard solutions, 2.5, 5, 7.5 *and* 10 AGU ml^{-1} of *NOVO enzyme in de-mineralised water*. The solutions are stable for up to 1 month if kept frozen at −15 °C in disposable centrifuge tubes.

Procedure

A diagram of the FIA procedure is shown in Fig. 1. The sample (30 μl) is injected into the carrier stream and the pNPG reagent is added. The incubation takes place at 50 °C, pH 4.3.

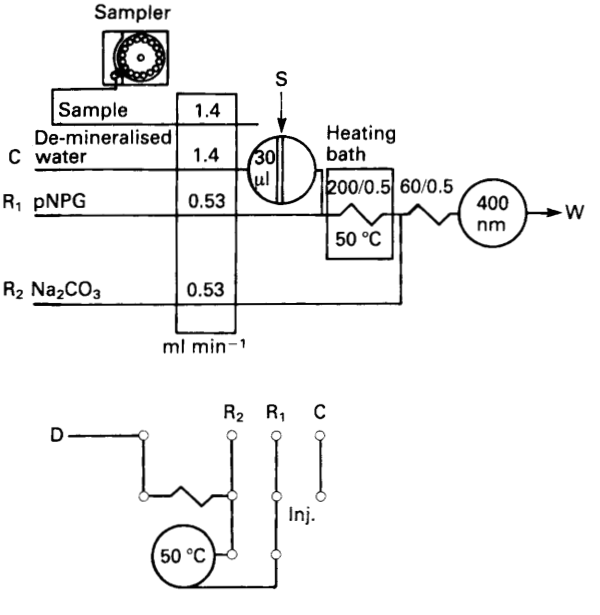

Fig. 1. Flow diagram for the FIA amyloglucosidase method

The enzyme reaction is stopped by adding sodium carbonate solution, resulting in a final pH of 10.1, and the *p*-nitrophenol is then determined spectrophotometrically at 400 nm.

Results

The starting point of the present FIA method is an unpublished manual analytical method,[6] which uses the following conditions: 1 ml of the sample (0.5 AGU ml^{-1}), 2 ml of pNPG (1 g l^{-1}) and an incubation time of 20 min (pH 4.3, 30 °C). The enzyme reaction is stopped by the addition of 3 ml of borax solution (0.1 M). The spectrophotometric determination of *p*-nitrophenol is then immediately carried out at 400 nm.

Incubation Temperature

With the manual method, the incubation time is 20 min at 30 °C. With FIA, the incubation time is extremely short (only 20 s), *i.e.*, it is the initial reaction rate of the enzyme that is registered. However, the degree of conversion of the *p*-nitrophenyl-α-D-glucopyranoside at the short incubation time is smaller than that observed with the manual method. To compensate for this, the manual incubation temperature of 30 °C was raised to 50 °C, giving an increase in sensitivity for the detection of the enzyme reaction products of 300%. This is possible as the NOVO amyloglucosides enzyme is heat-resistant up to 55–60 °C at pH 4.3 .

Incubation Time

In an attempt to increase the sensitivity of the method, the incubation time was increased by using the "stopped flow" facility of the Tecator 5020. At a time (T_1) after sample injection, when the sample was in the incubation coil, the flow was stopped at a previously fixed time (T_2). In the experiment the incubation time was increased from 20 s to 25, 30, 40 and 50 s total incubation time (Fig. 2). As was expected, a gain in sensitivity was obtained, but the calibration graph for enzyme was still rectilinear only up to an absorbance of 0.2 for 5 AGU ml^{-1} (T_2 = 0).

Incubation Buffer

Acetate buffer (pH 4.3, 0.1 M) was used as an incubation buffer. In order to determine the *p*-nitrophenol by spectrophotometry, it is necessary to change the pH from 4.3 to a basic value. The molar absorptivity for *p*-nitrophenol is dependent on pH up to pH 10.[8] The application of borate buffer (0.1 M), as indicated in the manual instructions, gave a pH of 9.1; however, when the borate buffer was replaced by a sodium carbonate buffer, a pH of 10.1 was recorded, which gave an additional gain in sensitivity of 5%.

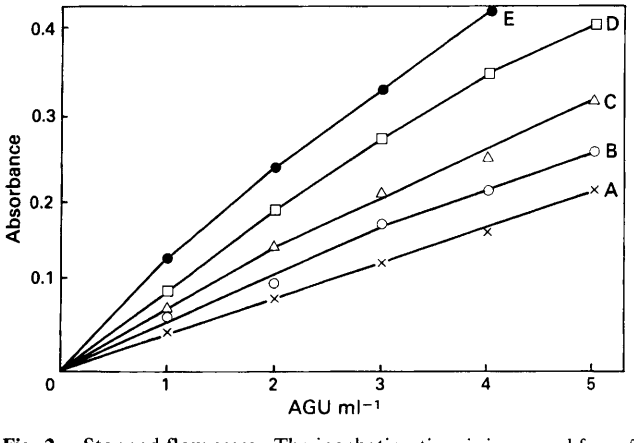

Fig. 2. Stopped flow assay. The incubation time is increased from 5 to 30 s (T_2):

	T_1/s	T_2/s
A	0	0
B	5	5
C	5	10
D	5	20
E	5	30

pNPG Reagent

The specificity of the analytical method is dependent on the purity of the *p*-nitrophenyl-α-D-glucopyranoside reagent. Certain commercial products may be contaminated with *p*-nitrophenyl-β-D-glucopyranoside. This is undesirable, because the β-glucosidase will also be determined by the assay. It is possible to determine the purity of the *p*-nitrophenyl-α-D-glucopyranoside in the following way: pure β-glucosidase may not give any reaction in a solution of *p*-nitrophenyl-α-D-glucopyranoside.

pNPG Concentration and Determination of K_m

Using the standardised conditions, the pNPG concentration was varied from 1 to 2 g l^{-1} (Fig. 3); 2 g l^{-1} gave the best response with regard to linearity and reproducibility. Using the Hanes plot, the Michaelis - Menten constant (K_m) was determined to 6 mM pNPG l^{-1}. As can be seen from Fig. 3, the optimum sodium carbonate concentration is 0.1 M.

p-Nitrophenol Spectral Curve

The maximum absorption of the reaction end product, *p*-nitrophenol, was examined at pH 7 and 11.4 (Fig. 4). At neutral pH, a maximum absorption was seen at 310 nm (A = 0.23), but a pH shift to 11.4 gave a very sharp maximum at 400 nm (A = 0.378).

Establishment of a Sample Blank Value

If the culture broths are very strongly coloured it is necessary to include a sample blank value. This may be done by changing the addition of reagent on FIA, so that the sodium carbonate reagent is added before the pNPG reagent.

Sensitivity of Method

With FIA, the enzyme can be determined from 0.5 to 10 AGU ml^{-1}, whereas the corresponding level of activity that can be determined by the manual method[6] is only 0.1 to 1 AGU ml^{-1}.

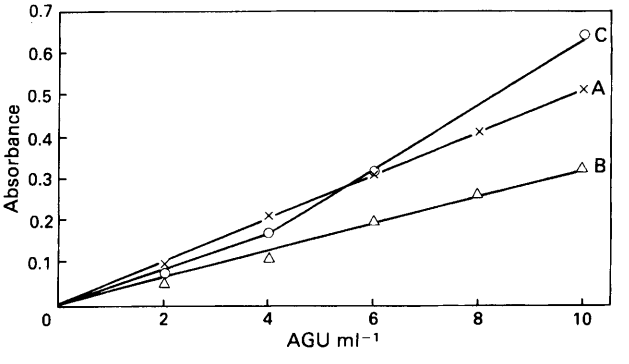

Fig. 3. Experiments with variation in pNPG concentration:

	pNPG/g l^{-1}	Na_2CO_3/M
A	2	0.1
B	1	0.1
C	2	0.5

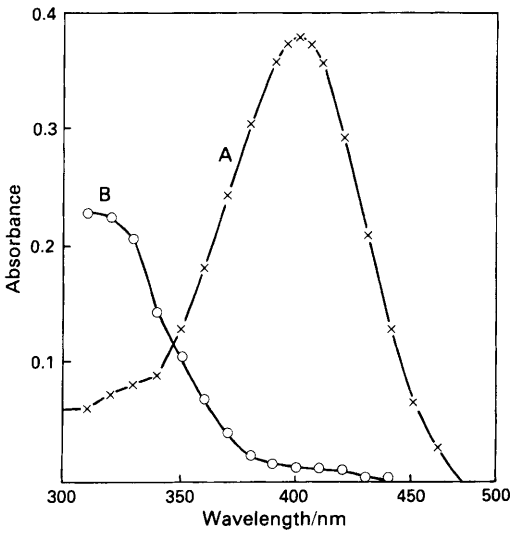

Fig. 4. Effect of pH on maximum absorption. pNP concentration, 20 μmol l^{-1}. pH: A, 11.4; and B, 7.0

Speed of Analysis

With a sampler coupled in, it is possible to analyse about 90 samples per hour. The present method has been used for 2 years in our laboratory with excellent results.

Discussion

In principle, the present FIA method is very flexible as it can be used for the determination of various hydrolytic enzymes, which are able to cleave the *p*-nitrophenyl compounds. The described method is based on the amyloglucosidase - *p*-nitrophenyl-α-D-glucopyranoside reaction, but has also, in our laboratory, been used for the determination of α-arabinofuranosidase with *p*-nitrophenyl-α-L-arabinofuranoside as the chromophore and α-galactosidase activity with *p*-nitrophenyl-α-D-galactopyranoside as the chromophore.

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