

Flow injection spectrophotometric assay of α -amylase activity

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Abstract

A spectrophotometric flow injection method for the determination of α -amylase activity is proposed. The method is based on the analysis of the maltose obtained during the hydrolysis of amylose in the presence of α -amylase and relies on the reaction of 3,5 dinitrosalicylic-acid and maltose at boiling temperatures to form a brick red coloured complex, monitored spectrophotometrically at 540 nm. The method has proved to be highly sensitive and rapid to allow analysis of 60 samples per hour. The % R.S.D. of this method was found to be <0.5% with the working activity range of 0.005–0.06 FAU. The detection limit was found to be 0.0048 FAU. (FAU: fungal amylase unit; 1 FAU is the amount of the enzyme that hydrolyses 5.26 g of starch per hour. 1 FAU=249.85 SIGMA). © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

α -Amylase is widely distributed in nature, i.e. in mammals, fungi, bacteria, most of the plant kingdom, etc. [1]. Its activity is very important in both clinical and food chemistry. In clinical chemistry the activity of α -amylase indicates the presence of parotitis, pancreatitis, and reveals occlusion of the pancreas. If present in saliva, it favoured the production of dental caries [2]. For food industry the hydrolysis of starch in the presence of α -amylase is important for the production of saccharin and glucose and is therefore extensively used in the sweet factory [3]. The amount of starch transformed into glucose and related sugars is linked to α -amylase activity. The production of ethanol also relies heavily on the usage of the hydrolysis product from α -amylase activity. The brewing and confectionery industries both depend on the activity

of α -amylase. Due to its high impact in diagnosis and in the quality of food it is important that the methods proposed for the assay of α -amylase activity should be characterised by objectivity and high selectivity. The need to obtain data with real time and effectively can be obtained by using flow injection analysis (FIA). The on-line measurement of the enzyme activity is very important especially for bioprocessing monitoring, where FIA has been established as a key tool [4]. Recktenwald et al. proposed in 1985 the on-line monitoring of enzymes in downstream processing by using spectrophotometric FIA [5]. An overview concerning the utilisation of FIA in the determination of enzyme activity is proposed by Luque de Castro and Fernadez-Romero [6].

Several techniques have been proposed for the assay of α -amylase [7–9]. High performance liquid chromatography [10] was used to sample starch in a study of the production of maltooligosaccharides by synergistic action of two enzymes. The correlation between salivary α -amylase isoenzymes and dental caries was studied by using capillary zone electrophoresis and

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hydrophobic-interaction chromatography [2]. The chromatographic techniques are usually labourious, time consuming, not suitable for on-line monitoring and have no sufficient precision and that is why a direct spectrophotometric method is preferred for the determination of the activity of α -amylase.

The utilisation of sensors [11] in FIA systems also improved the quality of analytical information [12,13]. A spectrophotometric technique based on the degradation of starch by α -amylase and measurement of the residual starch iodine-complex is also used in an FIA system [14]. Fourier-transform-infrared spectroscopy has been recommended as a detection method of a FIA system for the assay of the activity of α -amylase [15]. Min et al. [16] proposed a SIA technique for the assay of α -amylase activity. The detection is based on the monitoring of the de-colouration of the starch-iodine complex.

This paper proposes a new FIA system for the assay of α -amylase activity that is an automation of a standard method that is usually used for the determination of α -amylase activity [17]. Maltose is the major component of the amylase hydrolysis reaction. It occurs as 70% of the product and can thus safely be used as the indicator for the diastasis activity. The assay of α -amylase is based on the determination of maltose obtained in the hydrolysis of starch at 37°C [17]. 3,5-Dinitrosalicylic acid is selective for maltose and the other reducing sugars which are all products of starch digestion and can thus be reliably used as an indicator for α -amylase activity. The activity of an enzyme is changed when immobilised on a support [11,18,19] and it is necessary to adopt an FIA system where the enzymatic solution is injected into the system or by using the merging zone approach. Practically, the proposed system relies on the selective reaction of 3,5-dinitrosalicylic acid with maltose at high temperatures (96°C). The colour that results from the reaction between maltose and the acid develops fast enough to be effectively used in FIA.

2. Experimental

2.1. Reagents and solutions

All reagents used were prepared from analytical grade chemicals. Deionised water was used through-

out. The water was tested beforehand for any traces of other ions that may exist after treatment. All solutions were degassed before any measurements with a vacuum pump system. The main solutions were prepared as follows.

2.1.1. Chromogenic reagent

1 g of 3,5 dinitrosalicylic acid (Fluka, Buchs SG, Switzerland) was dissolved in 20 ml of 2 mol/l NaOH solution, followed by the addition of 30 g of sodium-potassium tartrate and diluted to 100 ml with deionised water. The solution was protected from CO₂ by covering with a tight fitting cap.

2.1.2. Phosphate buffer

9.470 g of Na₂HPO₄ (Merck, Germany) and 9.208 g of NaH₂PO₄ (Merck, Germany) were each dissolved in sufficient deionised water before they were individually diluted to 1 l. To prepare 500 ml phosphate buffer (pH 6.89), 200 ml of Na₂HPO₄ were mixed with 300 ml of NaH₂PO₄ and 2.3 g of dried NaCl. This was followed by the adjustment of the pH to 6.89 with either 2 mol/l HCl or 2 mol/l NaOH as required.

2.1.3. Starch

The starch solution was prepared by dissolving 0.270 g of dry starch (Merck) in 200 ml of 6.89 phosphate buffer (pH=6.89) and gently boiling for 1 min. The solution was slowly cooled to room temperature and stored at room temperature. New starch solutions were prepared when the sensitivity of the proposed system shows any kind of decrease in sensitivity. Phosphate buffer was used for the dilution of the solution to 400 ml.

2.1.4. α -amylase working standard solution

An α -amylase (from *Bacillus licheniformis*, 2.0 U/mg) (Fluka, EC 3.2.1.1, Buchs, Switzerland) solution was prepared by addition of the appropriate mass of the pure α -amylase to a specific volume of deionised water to prepare a standard stock solution (0.05 FAU). The other working α -amylase solutions (0.01–0.04 FAU) were obtained by serial dilutions from the standard stock solution. 1 FAU (fungal amylase unit) is the amount of enzyme that hydrolyses 5.26 g of starch per hour. The conversion factor from starch via α -amylase activity to maltose is 0.95 and therefore the amount of maltose formed is 4.997 g/h.

One SIGMA unit liberates 1 mg of maltose from starch in 3 min at pH 6.9; therefore 0.02 g maltose per hour. The factor between FAU and SIGMA units is therefore 1 FAU=249.85 SIGMA.

2.2. Apparatus

The following equipment was used: a Cenco peristaltic pump operating at 10 rpm; a VICI 10-port two-position sampling valve with two identical sampling loops, each having a volume of 50 ml; reaction manifold system (Fig. 1); three thermostat closed water baths set and carefully controlled at 37, 100 and 25°C; a Pye Unicam SP6-550 UV–VIS spectrophotometer equipped with a 10 mm Hellma-type flow-through cell (volume 80 μ l); PC30-B interface board (Eagle Electric, Cape Town, South Africa) for data acquisition and device control; a distribution board (MINTEK, Randburg, South Africa).

The FlowTEK [20,21] software package was incorporated into the system.

2.3. Flow system

The sequence of equipment interaction for the determination of α -amylase activity is represented in a detailed schematic outlay in Fig. 1. The manifold consists of Tygon tubing with an inside diameter of 1.14 mm cut into appropriate lengths and wound

around glass rods with an outside diameter of 15 mm. The manifold consisted of three tubes one leading to the valve system and propelling deionised water as carrier stream at 1.2 ml/min. 60 μ l samples were injected into the carrier stream. The next one propelled starch solution in phosphate buffer at 1.2 ml/min that mixed with the sample before leading into a 37°C water bath. The last tube propelled the colour reagent at 2.5 ml/min to mix with the hydrolysis product before leading into a 96°C water bath. (Fig. 1).

The main reaction (catalysed by α -amylase) is taking place in the first bath at 37°C. The reaction between maltose and 3,5 dinitrosalicylic acid occurred at the high temperature of 96°C. The final product was cooled to 25°C in a water bath before monitored spectrophotometrically at 540 nm.

2.4. Sample preparation

Seeds were germinated for 3 days, then harvested and dried in an oven. They were then ground and 2% peptone solution was added, followed by stirring for 2 h, centrifugated and filtered using Whatman filter paper. The supernatant was collected and analysed for α -amylase activity. For powdered samples the sample preparation method was followed after the addition of 2% peptone solution and carried on from this step as described above for seed treatment.

3. Results and discussion

3.1. Optimisation of the method

The flow injection (FI) parameters have been optimised univariately. Although multivariate techniques are sometimes preferred, optimisation of physical parameters like residence times as functions of tube lengths, inner diameters, etc. are more expensive and in this study the results from univariate optimisation were sufficient for the goal set. In order to obtain a reliable assay of α -amylase activity, the following parameters were optimised.

3.1.1. Effect of flow rates

A series of preliminary and evaluation trials were conducted in order to optimise the flow rates of the colour, carrier and starch solutions. The experiments

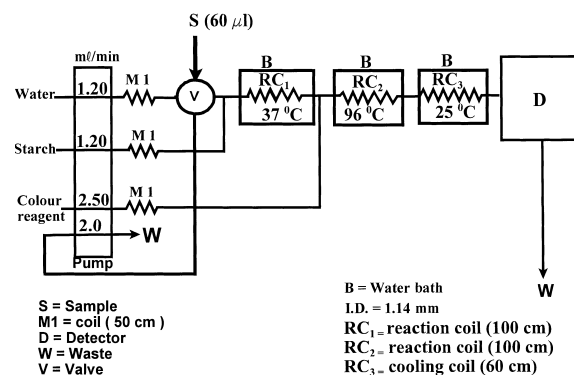


Fig. 1. Schematic flow diagram for the determination of α -amylase activity. M: mixing coil; D: detector; V: injection valve; W: waste; M1=50 cm; S: sample; RC₁: reaction coil 1 (hydrolysis region); RC₂: reaction coil 2 (colour development region); RC₃: cooling region.

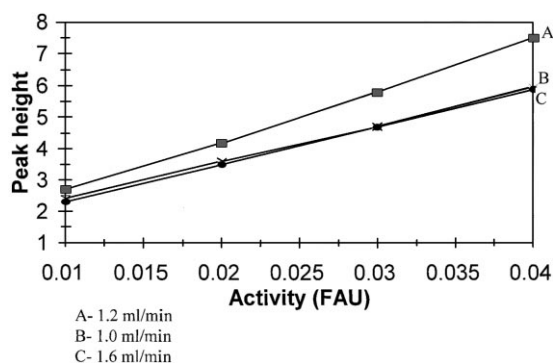


Fig. 2. Effect of carrier-starch flow rate at constant colour reagent flow rate (2.5 ml/min).

proved that the method performs better when the flow rates of the carrier and starch were similar and that the total of both these flow rates was almost equal to that of the colour reagent. This leads to either varying the starch-carrier flow rate and keeping the flow rate for colour constant or vice versa.

3.1.2. Effect of starch flow rate when a constant colour reagent flow rate (2.5 ml/min) is used

Fig. 2 displays the effect of the flow rate of starch solution. It is evident from Fig. 2 that a flow rate of 1.2 ml/min gives a maximum sensitivity and a straight calibration curve. As stated earlier the system operated better when the total starch-carrier flow rate was almost equal the colour flow rate. Therefore, a total of 2.4 ml/min feeds the system with a steady volume of the reagents which is stoichiometrically sufficient for amylase hydrolysis and colour development.

3.1.3. Effect of colour reagent flow rate when a constant starch flow rate (1.2 ml/min) is used

A flow rate of 2.5 ml/min for the colour reagent yielded the best sensitivity and precision with a straight calibration curve (Fig. 3). With higher flow rates the residence time of reagents in the reaction coil (RC_2) decreases to such an extent that the 3,5-dinitrosalicylic acid has not sufficiently bound to the maltose for full colour development and the response dropped as seen from Fig. 3. The sensitivity and also precision decreased for lower flow rates due to insufficient reagents to react with the colour reagent as only minimal quantities will be propelled.

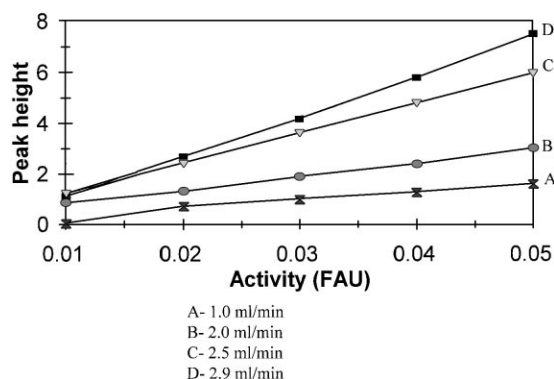


Fig. 3. Effect of colour reagent flow rate at constant carrier-starch flow rate (1.2 ml/min).

3.1.4. Influence of the different reaction coil lengths

There are basically three different operating conditions at different temperatures involved in the flow injection spectrophotometric assay of α -amylase activity. As each temperature has a different function to fulfill, care should be taken to use the optimum working temperature, to make sure that this temperature should be reached as fast as possible and that the optimum product is formed. The main reaction, that is catalysed by α -amylase, is the hydrolysis of the 1,4-glycosidic bond from starch. The optimum operational temperature for the α -amylase activity is body temperature of 37°C. The residence time as a function of reaction coil 1 length (RC_1) was evaluated and it was found that a reaction coil of 100 cm gave the best sensitivity and precision. The second reaction between maltose and 3,5-dinitrosalicylic acid is temperature dependent and is much more pronounced at 96°C. The following parameters play a very important role in this part of the reaction and were evaluated: the reaction should reach 96°C as fast as possible, residence time and dispersion that is dependent on coil length. The reaction coils were wound around solid rods and also around hollow tubes with outside diameters of 15 mm. Fig. 4 displays this trend of results, however a hollow support for the reaction coil seemed to yield the best results as it exposes the reaction coil to a larger volume of hot water at 96°C than does a solid cylindrical support. A reaction coil (RC_2) of 100 cm on a hollow support gave the best response and precision and was used for further work. The formation and accumulation of air bubbles in the flow cell is one of the main problems

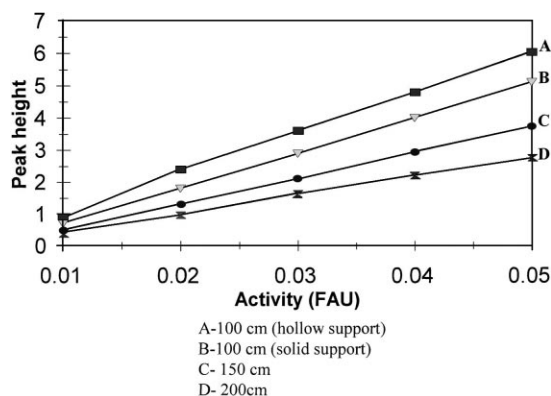


Fig. 4. Effect of reaction coil length.

for routine use of the proposed flow injection system. At the temperature of 96°C the system is more prone for this. It was therefore necessary to cool down the manifold to 25°C before detection. It was found that a 60 cm cooling coil was sufficient enough for this purpose.

3.1.5. Effect of NaOH addition into the colour reagent solution

Through preliminary experiments it was discovered that the addition of 2 mol/l NaOH as percent fraction of the total volume of colour reagent solution had a marked influence on the sensitivity of the reaction between the maltose and the 3,5-dinitrosalicylic acid. Various volume fractions of the 2 mol/l NaOH as fraction of the total volume of the colour reagent solution were evaluated and the results are given in Fig. 5. The

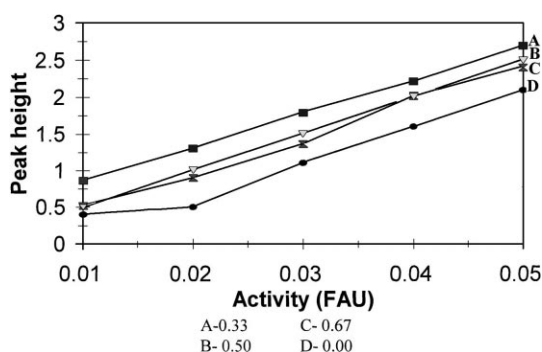


Fig. 5. Effect of 2 mol/l NaOH solution.

Table 1

Effect of starch concentration (% w/w) on the peak height for different α -amylase activity (FAU)

Starch concentration % (w/w)	α -Amylase activity (FAU)				
	0.01	0.02	0.03	0.04	0.05
0.01	1.099	2.711	4.203	5.803	7.409
0.1	2.842	4.605	6.403	8.205	–
0.15	2.961	4.878	6.510	8.300	–
1	3.026	5.884	8.009	–	–

best results were obtained with a 0.33% (v/v) NaOH and this was used for further work.

3.1.6. Effect of starch concentration

The concentration of starch has a significant bearing on the sensitivity of this method. It also gives the working range for this analysis since it is the substrate for this enzymatic reaction. Enzymatic studies proved that the substrate should always be in excess compared with the enzyme concentration. It was determined that 0.1 and 1% gave results that went beyond the chosen sensitivity range for some chosen amylase concentration. A 0.01% (w/w) of starch was chosen after it showed consistent results for all the standards (Table 1). Once the starch solution has been prepared, it is not exposed to high temperatures, but only to 37°C which is the optimum temperature for the α -amylase activity and the same temperature used for the standard method. Compared to the manual method the period of exposure for the proposed FIA system is relatively small and also more repeatable due to the hydrodynamic nature of the FIA system and also not allowing any substantial change to occur in the form of the substrate. The product of starch breakdown is the one that is exposed to a high temperature for colour development.

3.1.7. Effect of colour reagent concentration

Maltose, the major component of the α -amylase hydrolysis reaction, and 3,5 dinitrosalicylic acid reacts at high temperatures to form a brick red coloured complex that is monitored spectrophotometrically at 540 nm. Therefore, the influence of the concentration of a 3,5 dinitrosalicylic acid solution in 2 mol/l NaOH were evaluated and the results are given in Table 2. Although the best response was obtained with a 1.5% (w/w) 3,5 dinitrosalicylic acid (Table 2), the

Table 2

Effect of colour reagent concentration on the peak height for different α -amylase activity (FAU)

Colour reagent concentration % (w/w)	α -Amylase activity (FAU)				
	0.01	0.02	0.03	0.04	0.05
0.1	0.381	0.718	0.815	0.921	1.286
0.5	0.483	0.888	1.012	1.524	1.834
1	1.099	2.711	4.203	5.803	7.409
1.5	0.634	1.090	1.698	2.070	2.788
2	0.534	0.912	1.310	1.785	2.011

best linearity and precision was obtained with a 2% (w/w) concentration and this was used for the proposed system.

3.1.8. Influence of temperature of reaction coil (RC_2)

Temperature is a major role player in the reaction between maltose and 3,5 dinitrosalicylic acid. The influence of temperature was evaluated and it was found that the response increase dramatically when the temperature is raised up to 96°C (Table 3) and this temperature was used throughout.

3.2. Evaluation of the method

The sensitivity and performance of the method were evaluated under optimised conditions with regards to detection limit, linearity, accuracy, precision, sample interaction, and sample rate.

3.2.1. Detection limit

It is very important that any new method should be evaluated for its detection limit to be sure which concentrations of the analyte can be determined with confidence and that the results obtained can be trusted. The detection limit for the method was calculated

using the following formula:

$$DL = \frac{[3S_b + I_b] - k}{m}$$

where DL is the limit of detection, S_b denotes the standard deviation of the background signal, I_b represents the relative peak height of the background signal; k the intercept of the calibration graph and m is the slope of the calibration graph.

The detection limit was found to be 0.0048 FAU

3.2.2. Linearity

The linearity of the method was evaluated under optimum conditions for the system. The data collected for the standards yielded a straight line between 0.01 and 0.05 FAU. The regression coefficient for this system is

$$H = -0.47 + 157.12a, \quad r = 0.9998$$

where H is relative peak height and a is the enzymatic activity (FAU). The correlation coefficient (r) indicated that the method is linear for the chosen working standards.

3.2.3. Precision

The precision for the proposed FIA system was tested by performing ten repetitions of the analysis on each of the different samples (Table 4) and the % RSD was found to be less than 0.5%.

3.2.4. Accuracy

The results from analysis by the proposed system were tested for accuracy by comparing them with those from well established and standard methods, which are the iodine and the falling number methods. It is evident from Table 4 that these results did not differ much from those obtained with the other methods and that the results compared excellently.

3.2.5. Sample interaction

The influence of sample interaction seemed to be insignificant as there was no observable change or shift from the baseline with each successive analysis. This is due to the fact that at some stage the method employs very high temperature which aids in the cleaning of the system. However, it was necessary to rinse the system with copious amounts of the buffer after the analysis

Table 3

Effect of temperature of the colour development water bath on the peak height for different activities of α -amylase

Temperature (°C)	α -Amylase activity (FAU)				
	0.01	0.02	0.03	0.04	0.05
60	0.368	0.399	0.431	0.451	0.457
80	0.477	0.512	0.610	0.610	0.645
96	1.099	2.711	4.203	5.803	7.409

Table 4

Comparison between the results obtained for the α -amylase activity (FAU) with the proposed FIA method and with two standard methods [17]

Samples	Proposed FIA method	FIA iodine method	Falling no. method
Malt wheat samples	0.034 $\pm$ 0.015	0.035 $\pm$ 0.030	0.032 $\pm$ 0.027
	0.037 $\pm$ 0.014	0.039 $\pm$ 0.033	0.034 $\pm$ 0.030
	0.031 $\pm$ 0.011	0.032 $\pm$ 0.029	0.028 $\pm$ 0.026
Malt sorghum samples	0.006 $\pm$ 0.002	0.006 $\pm$ 0.005	0.006 $\pm$ 0.003
	0.008 $\pm$ 0.003	0.008 $\pm$ 0.006	0.008 $\pm$ 0.005
	0.005 $\pm$ 0.001	0.006 $\pm$ 0.004	0.005 $\pm$ 0.003
Energy food morvite samples	0.042 $\pm$ 0.012	0.044 $\pm$ 0.023	0.042 $\pm$ 0.026
	0.048 $\pm$ 0.011	0.047 $\pm$ 0.032	0.048 $\pm$ 0.035
	0.043 $\pm$ 0.012	0.043 $\pm$ 0.022	0.044 $\pm$ 0.029

was completed and just before another batch could be determined.

3.2.6. Sampling rate

The proposed method is faster than the other FIA methods presented in the literature [12,13]. The sampling rate was found to be 60 samples per hour.

4. Conclusion

The proposed flow injection analysis method satisfies all the requirements for an analytical method. It is reliable with precision and accuracy clearly demonstrated. When compared to other known and tried methods it stands out as a simple and fast one. The enzyme is not immobilised on a support, therefore the enzymatic activity determined is accurate. The precision obtained is better than the one given by the other FIA systems [12,13].

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