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The effect of carbohydrates on α-amylase activity measurements

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Abstract

The Ceralpha method can be used for α-amylase activity measurements during the hydrolysis of starch at high substrate concentrations (>40 wt.%). However, the results are affected by the carbohydrates present in the samples. The effect of carbohydrates on the Ceralpha α-amylase activity measurements was measured over a broad concentration range. It was found that starch has the largest influence and glucose has the lowest influence on the Ceralpha assay procedure. These results were explained by considering substrate inhibition and substrate competition. A simple kinetic model was used to describe the observed phenomena quantitatively. This model was also used to estimate the Michaelis–Menten constant for a large number of substrates and it requires only a single experiment for each K_m determination.

Keywords: α-Amylase activity assay; Ceralpha method; Carbohydrates; Kinetic model; Substrate inhibition; Substrate competition; Michaelis–Menten constant

1. Introduction

One of the most important enzymatic reactions that is carried out at an industrial scale is the enzymatic hydrolysis of starch. α-Amylase (1,4-α-D-glucanohydrolase, EC 3.2.1.1) is essential during this process and plays a role in the liquefaction of starch and the subsequent saccharification where larger carbohydrate chains are hydrolysed and converted into smaller carbohydrates [1]. It is, therefore, important to preserve the highest α-amylase activity during industrial operating conditions.

Currently, enzymatic starch hydrolysis is carried out at a substrate concentration of 35–40 wt.% [2]. Increasing the substrate concentration has several advantages such as increased volumetric productivity and a higher α-amylase stability [3–5]. However, the influence of these conditions on the enzyme activity also needs to be considered. A suitable assay procedure needs to be chosen to determine the residual α-amylase activity under these operating conditions.

Several assay procedures exist and they can be divided into three groups: reducing sugar methods, dyed starch substrate methods and defined substrate methods [6]. Working at high substrate concentrations leads to a high amount of reducing ends in the samples containing α-amylase and it is, therefore, difficult to achieve sufficient resolution. For this reason, assay procedures based on reducing sugars are not desirable. Furthermore, the addition of more starch is also not advisable because it increases the viscosity of the samples even further. Therefore, a suitable defined substrate method is preferred. We chose for the Ceralpha method that was developed by McCleary and Sheehan [7,8] that is now commercialised by Megazyme. This is a rapid and accurate assay [9] that is assumed to be suitable for samples with high background levels of small carbohydrates.

The Ceralpha procedure employs Amylase HR reagent that contains a defined oligosaccharide 'non-reducing-end blocked *p*-nitrophenyl maltoheptaoside' in the presence of excess levels of a thermostable α-glucosidase. During the assay, endo α-amylase cleaves a bond somewhere in this defined oligosaccharide and due to the excess quantities of α-glucosidase present in the mixture the remaining *p*-nitrophenyl maltosaccharide is hydrolysed very rapidly to glucose and free *p*-nitrophenol. The amount of *p*-nitrophenol released is a measure for the α-amylase activity. The release of this compound leads to a yellow colour that can be measured with a spectrophotometer.

The Ceralpha method from Megazyme is widely used, however, the influence of different carbohydrates on the outcome of this α-amylase activity assay has never been reported to our knowledge. In order to study this, we chose for components that are also present in samples from hydrolysis experiments, e.g. soluble starch and various starch hydrolysates. A simple kinetic model describing substrate inhibition and substrate competition

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was used to explain and describe our observations. Furthermore, the same kinetic model was used to calculate estimates of Michaelis–Menten parameters of other substrates.

2. Materials and methods

2.1. Materials

Thermostable α-amylase from *Bacillus licheniformis* (EC 3.2.1.1, Termamyl 120L, type XII-A) was obtained from Sigma–Aldrich (Steinheim, Germany). The enzyme concentration used in the experiments is expressed in grams of this enzyme stock solution per liter. Anhydrous D(+)-glucose, maltotriose, maltodextrin from corn with dextrose equivalents of 4–7, 13–17 and 16.5–19.5 and soluble potato starch were obtained from Sigma–Aldrich (Steinheim, Germany). Maltose monohydrate, sodium chloride, calcium chloride dihydrate and tri-sodium phosphate were bought from Merck (Darmstadt, Germany). Maltotetraose, maltopentaose, maltoheptaose and maltohexaose were present in an oligosaccharide kit obtained from Supelco (Bellefonte, PA, USA). Maleic acid (di-sodium salt) was obtained from Acros Organics (Geel, Belgium). For the measurement of the α-amylase activity Amylase HR reagent was used and this is a product form Megazyme International Ireland (Bray, Republic of Ireland). The standard buffer used for all the experiments was 0.1 M maleic acid buffer (pH 6.5) with 2 mM $CaCl_2$ and 0.1 M NaCl. A solution of 0.06 M tri-sodium phosphate (pH 11) was used as stopping reagent during the Ceralpha end-point measurements.

2.2. α-Amylase activity

The α-amylase activity is defined as the amount of *p*-nitrophenol released in $\mu mol\,mg^{-1}\,min^{-1}$ of enzyme solution at 40 °C and pH 6.5. To determine this activity, we calculated the release of *p*-nitrophenol by measuring the absorbance of the reaction mixture at 410 nm after incubation at 40 °C (end-point method) and pH 6.5:

$$A_E = \frac{A - A_0}{\Delta t}\frac{1}{\varepsilon_1}\frac{1}{b}\frac{1}{C_E}D \quad (1)$$

where A_E is the α-amylase activity in $\mu mol\,mg^{-1}\,min^{-1}$, A the absorbance at 410 nm and 25 °C, A_0 the absorbance of the blank solution at 410 nm and 25 °C (reaction mixture before incubation), Δt the incubation time in min, ε_1 the molar absorptivity at 410 nm, 25 °C and pH 11 in $l\,\mu mol^{-1}\,cm^{-1}$, b the path length of the light in the cuvette in cm, C_E the enzyme concentration in $mg\,l^{-1}$ and D is the dilution factor.

The release of *p*-nitrophenol as a result of enzymatic break down by α-amylase can also be determined by continuous measurement of the absorbance of the reaction mixture at 410 nm, 40 °C and pH 6.5:

$$A_E = \frac{dA}{dt}\frac{1}{\varepsilon_2}\frac{1}{b}\frac{1}{C_E}D \quad (2)$$

where dA/dt is the slope of the linear part of the absorbance–time curve and ε_2 is the molar absorptivity at 410 nm, 40 °C and pH 6.5 in $l\,\mu mol^{-1}\,cm^{-1}$. The continuous activity measurements have the advantage that the linear part of the graph can always be selected. The lag phase that is often observed [10] during the beginning of the assay can be circumvented in this way. Determination of the activity with the original Ceralpha end-point method does not enable a correction of the lag phase and this makes it less accurate. However, the advantage of the end-point method is that the residual α-amylase activity can be determined in a large number of samples at once.

2.3. Measurement of molar absorptivity

The molar absorptivity of *p*-nitrophenol depends strongly on pH and temperature [10] and to take this into account we determined the molar absorptivity of *p*-nitrophenol at our measurement conditions. Stock solutions with a *p*-nitrophenol concentration varying between 7.2×10^{-6} and $4.9 \times 10^{-5}\,mol\,l^{-1}$ were made in stopping reagent/maleic acid buffer (15/2 v/v). A second set of stock solutions was made with a *p*-nitrophenol concentration varying between 1.4×10^{-5} and $9.7 \times 10^{-5}\,mol\,l^{-1}$ in maleic acid buffer/demineralized water (7/3 v/v). The pH of these stock solutions were equal to pH of the solutions that were used during the experiments. The molar absorptivity for *p*-nitrophenol in stopping reagent/maleic acid buffer (pH 11) at room temperature is $19.5 \times 10^{-3}\,l\,\mu mol^{-1}\,cm^{-1}$ and in maleic acid buffer/demineralized water (pH 6.5) at 40 °C it amounts to $8.0 \times 10^{-3}\,l\,\mu mol^{-1}\,cm^{-1}$.

2.4. Measurement of K_m and V_{max} for blocked p-nitrophenyl maltoheptaoside

A $0.32\,g\,l^{-1}$ α-amylase stock solution, Amylase HR reagent and maleic acid buffer were separately pre-incubated for 10 min at 40 °C in a water bath. After pre-incubation 100 μl of α-amylase stock solution was added to respectively 10, 30, 50, 70, 100, 200, 300, 400, 500 and 800 μl of Amylase HR reagent. The volume was filled up to 1.7 ml with maleic acid buffer and incubated for 10 min at 40 °C in a spectrophotometer while measuring the absorbance at 410 nm. With these data, the initial reaction rates can be found at various concentrations of blocked *p*-nitrophenyl maltoheptaoside. The computer program 'Table Curve 2D' (Jandel Scientific, 1994, version 2.0, San Rafael, CA, USA) was used to determine the values of K_m and V_{max} by minimizing the sum of squared residuals. The enzyme solution that was used for the preparation of the stock solution for these measurements originated from a different batch than the enzyme solution that was used for the other measurements.

2.5. α-Amylase activity with different carbohydrates

Stock solutions of different carbohydrates (D(+)-glucose, maltodextrin and soluble starch) and with varying carbohydrate concentrations (between 0 and $286\,g\,l^{-1}$) in maleic acid buffer were made. Enzyme stock solutions for the continuous method contained 35 mg enzyme/(l solution) and for the end-point method they contained 480 mg enzyme/(l solution). For each set of experiments, a fresh enzyme stock solution was prepared. This solution was kept in the refrigerator for 2 h before an experiment was carried out.

The α-amylase activity in the starch solutions was measured according to the Ceralpha end-point assay procedure of Megazyme. The starch stock solutions ($0–45\,g\,l^{-1}$) were first heated to 95 °C and they were kept at this temperature for 1 h in order to gelatinize all the starch. After this treatment, the starch was cooled to 40 °C. The starch stock solutions, the enzyme stock solution and the Amylase HR stock solution were pre-incubated separately for 10 min at 40 °C in a water bath. After this pre-incubation period 9 ml of the starch stock solutions and 1 ml of the enzyme stock solution were added together and 100 μl of this mixture was added to 100 μl Amylase HR reagent. After an incubation period of exactly 10 min at 40 °C, the reaction was stopped by adding 1.5 ml of stopping reagent (pH 11) followed by the measurement of the absorbance at 410 nm and 25 °C. The α-amylase activity in different maltodextrin solutions was also measured with the Ceralpha end-point assay procedure of Megazyme. In this case, the gelatinization step was not necessary and therefore omitted.

The α-amylase activity in all carbohydrate solutions except the starch solutions was measured using the continuous method. The carbohydrate stock solution, the enzyme stock solution and the Amylase HR stock solution were pre-incubated separately for 10 min at 40 °C in a water bath. After pre-incubation equal volumes of the carbohydrate stock solution and the enzyme stock solution were added together. From this solution, 700 μl were taken and added to 300 μl Amylase HR reagent and incubated for 10 min at 40 °C in a spectrophotometer while measuring the absorbance at 410 nm. All the experiments were carried out in duplicate.

2.6. Determination of K_m values for different substrates

A carbohydrate stock solution that contained $100\,g\,l^{-1}$ of glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose or maltodextrin in maleic acid buffer (pH 6.5) and an α-amylase stock solution, which contained $30\,mg\,l^{-1}$ in the same buffer, were prepared. The continuous method described in the previous section was used for the activity measurements. All experiments were repeated five times (in case of maltotetraose, maltopentaose, maltoheptaose and maltohexaose) or six times (in case of glucose, maltose, maltotriose and maltodextrin).