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Inhibition of human α-amylase by dietary polyphenols

Hilda Nyambe-Silavwe [1], *Jose A. Villa-Rodriguez* [1], *Idolo Ifie* [1], *Melvin Holmes, Ebru Aydin, Jane Møller Jensen, Gary Williamson* *

School of Food Science and Nutrition, University of Leeds, Leeds LS2 9JT, UK

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ABSTRACT

Functional foods offer the possibility to modulate the absorption of sugars, leading to benefits for diabetics and those with metabolic syndrome. As part of the characterisation of such foods, inhibition of α-amylase is used to assess components for their potential ability to modify the post-prandial glycaemic response. Many publications on phenolics as potential inhibitors report widely varying assay conditions leading to variable estimates of inhibition. On this basis, we have optimised the *in vitro* α-amylase inhibition assay and, in particular, we show the importance of removing certain polyphenols after the enzymic reaction when using 3,5-dinitrosalicylic acid since they interfere with this reagent. There was a substantial ~5-fold effect on acarbose IC_{50} values when working just outside optimal conditions. This shows that inappropriate assay conditions, such as excess enzyme, greatly influence IC_{50} values and could explain some discrepancies in the existing literature.

1. Introduction

It is estimated that about 346 million people worldwide suffer from type 2 diabetes due to pancreatic β-cell dysfunction and/or increased resistance to insulin with impaired glucose tolerance (Danaei et al., 2011). The risk of developing impaired glucose tolerance is increased by regular high postprandial glucose spikes in the blood (Livesey, Taylor, Hulshof, & Howlett, 2008; Manzano & Williamson, 2010). Hydrolysis of starch is one of the main sources of postprandial glucose in the blood, with the enzymes α-amylase and α-glucosidase being involved in starch breakdown. Salivary and pancreatic α-amylases hydrolyse starch to produce maltose and other oligosaccharides by breaking the α-1,4 glycosidic bonds (Hanhineva et al., 2010; Williamson, 2013). Subsequently, the α-glucosidases located in the brush-border surface membrane of intestinal cells hydrolyse the resulting oligosaccharides into glucose, which is then transported into the blood by the transporters sodium dependent glucose transporter type 1 (SGLT1; SLC5A1) and glucose transporter type 2 (GLUT2; SLC2A2) (Scheepers, Joost, & Schurmann, 2004). Drugs such as acarbose (Supplementary Fig. S1) are used in the management of type 2 diabetes and act by inhibiting α-amylase and α-glucosidases. Other small molecules such as polyphenols (Supplementary Fig. S1) might have acarbose-like effects (Hanhineva et al., 2010; Williamson, 2013), and so could provide a suitable strategy to manage type 2 diabetes, since acarbose commonly causes side

* *Corresponding author.* School of Food Science and Nutrition, University of Leeds, Leeds, LS2 9JT, UK. Tel.: +44 113 343 8380; fax: +44 113 343 2982.
E-mail address: g.williamson@leeds.ac.uk (G. Williamson).
[1] These authors contributed equally to the article.
Abbreviations: DNS, 3,5-Dinitrosalicylic acid; EGCG, (-)-epigallocatechin gallate; PBS, phosphate buffer saline; SPE, solid phase extraction; SGLT1, sodium dependent glucose transporter type 1; GLUT2, glucose transporter type 2
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effects including flatulence, diarrhoea and nausea. Functional foods could ultimately be developed containing components able to inhibit α-amylase, an acarbose-like action but without the side effects.

Many reports (Tables 1–3) indicate that polyphenols inhibit α-amylase. However, these studies use different methods of detection and assay conditions (reaction time and temperature, pH, enzyme concentration and source, substrate concentration and source) which have a pronounced impact on the reported data. Acker and Auld (2014) recently outlined the importance of experimental conditions when designing enzyme assays in general. The most commonly used method for measuring α-amylase activity involves the DNS reagent for detection of reducing sugars. The presence of a free carbonyl group — in reducing sugars enables them to participate in an oxidation-reduction reaction with 3,5-dinitrosalicylic acid (DNS). However, due to the reducing potential of the polyphenols, we postulated that they could interfere with the development of the colour and therefore the results of the assay.

In this paper we report optimisation of the critical steps, showing the conditions required to assess α-amylase inhibition, using DNS as the detection method, and compare the measurement of *Ki* (the dissociation constant of the enzyme-inhibitor complex) and IC_{50} (concentration of inhibitor giving 50% inhibition) values for the potent inhibitor (-)-epigallocatechin gallate (EGCG).

2. Materials and methods

2.1. *Reagents and standards*

3,5-Dinitrosalicylic acid, potassium sodium tartrate, chromatographically purified human salivary α-amylase type IX-A, maltose, EGCG, quercetin, amylose and amylopectin from potato were all purchased from Sigma-Aldrich. Co., Ltd., Dorset, UK. Phloridzin, quercetin-3-*O*-glucoside and luteolin were purchased from Extrasynthese, Genay, France. Gallic acid was obtained from Alfa Aesar, Lancashire, UK. Instant green tea was obtained from Nestle Research Center, Lausanne, Switzerland. Oasis MAX cartridge 1 mL (30 mg) and 3 mL (60 mg) were purchased from Waters Corporation Ltd., Milford, MA, U.S.A. The DNS reagent was prepared by adding to 12 mL water, 20 mL of 96 mM DNS in water and 5.3 M sodium potassium tartrate solution (12 g in 8 mL of 2 M sodium hydroxide). For α-amylase, 1 "Sigma-defined" unit will liberate 1.0 mg of maltose from starch in 3 min at pH 6.9 at 20 °C, and this is the basis of our initial experiments to optimise the assay. The enzyme preparation on this basis contained 276 Sigma-units per mg protein by Bradford assay. All the reagents were of the highest purity and standards were ≥98 %.

2.2. *Enzyme concentration and reaction time*

Enzyme concentration and reaction time were determined by using different enzyme concentrations (0.5, 1.0, 1.5 and 2.0 U/mL), and assay mixtures were incubated for different times (0, 3, 6, 9, 12 and 15 min). The linearity of plots of absorbance at 540 nm (PHERAstar FS microplate reader, BMG Labtech, Inc., Cary, NC, USA) versus time was assessed.

2.3. *Determination of Km and Vmax*

The kinetic parameters K_m and V_{max} were determined by using a chosen enzyme concentration and incubation times giving linear rates of reaction. The substrate concentrations ranged from 0 to 1 mg/mL in the final assay volume. Maltose standard curve was obtained by adding 1 mL of the DNS reagent to a total volume of 500 μL of different maltose concentrations (0–2 mM) and then heated (100 °C for 10 min). The absorbance was recorded at 540 nm and the amount of maltose produced was calculated against the standard curve. The Lineweaver–Burk plot was used to calculate K_m and V_{max}.

2.4. *Effect of polyphenols on colour reagent*

The effect of polyphenols on the DNS reagent was determined by adding 1 mL DNS to an assay mixture containing 450 μL phosphate buffer saline (PBS, 0.01 M, pH 6.9) and 50 μL of different concentrations of the different polyphenols (0–1 mM). The absorbance was recorded as described previously.

2.5. *Retention efficiency of solid phase extraction cartridges by HPLC-PDA*

HPLC analysis for efficiency of retention of polyphenols by the Oasis MAX SPE cartridge was carried out with EGCG using a $UFLC_{XR}$ Shimadzu system (Shimadzu, Japan) consisting of binary pump, a photodiode array with multiple wavelength SPD-20A and an LC-20AD Solvent Delivery Module coupled with an online unit degasser DGU-20A3/A5 and a thermostat autosampler/injector unit SIL-20A (C). The column used was a 5 μm Gemini C_{18} (250 × 4.6 mm, i.d.) with a flow rate of 1 mL/min, column temperature set at 35 °C with an injection volume of 10 μL and detection at 280 nm. A two-phase gradient system consisting of water (Millipore grade) with 0.1% trifluoroacetic acid (HPLC grade) as mobile phase A and acetonitrile containing 0.1% trifluoroacetic acid as mobile phase B was used. The gradient conditions were as follows: initial conditions started with 92% A and increasing to 18% solvent B at 3.50 min, 32% B at 18 min, 60% B at 28 min reaching to 100% B at 32 min for 4 min, returning to the initial conditions for 3.5 min.

2.6. *α-Amylase inhibition assay*

The assay contained 200 μL each of substrate (amylose or amylopectin) and enzyme, 50 μL PBS and 50 μl of inhibitor of different concentrations. For the control assay, the inhibitor was replaced by an equal volume of PBS. Stock amylose and amylopectin solutions (2.5 mg/mL) were prepared in water by heating at 90 °C on a hot plate for 15 min. A second stock solution of amylopectin was prepared at 0.925 mg/mL. Human salivary α-amylase stock solution (1.25 U/mL) was prepared in PBS. The enzyme stock solution and the assay mixture containing the inhibitor, PBS and substrate were pre-incubated at 37 °C in a water bath for 10 min, and the reaction was started by adding the enzyme to the assay solution. The reaction was