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Applicability and reliability of the glucose oxidase method in assessing α-amylase activity

Rizliya Visvanathan[a,b], Chathuni Jayathilake[a], Ruvini Liyanage[a,*], Ramiah Sivakanesan[c]

[a] Division of Nutritional Biochemistry, National Institute of Fundamental Studies, Hantana Road, Kandy, Sri Lanka
[b] Postgraduate Institute of Science, University of Peradeniya, Sri Lanka
[c] Department of Biochemistry, Faculty of Medicine, University of Peradeniya, Sri Lanka

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ABSTRACT

Glucose oxidase (GOD) is an enzyme widely used in glucose monitoring systems owing to its high specificity towards glucose. However, in our previous work maltose was found to show significant interaction with GOD and based on this observation, a novel microplate-based method was developed to assess α-amylase inhibitory activity (GOD method). Concerns regarding the interaction of GOD with maltose has limited the widespread use of the GOD method in assessing α-amylase activity. The present paper provides answers to concerns regarding the interaction of GOD with maltose using HPLC studies and application of the GOD method in assessing α-amylase activity. According to the results, the newly developed GOD method can be considered as a well-suited method for the determination of α-amylase activity and as an easy method to do kinetic studies compared to other available methods.

1. Introduction

Glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase; EC 1.1.2.3.4) belonging to the family of glucose-methanol-choline (GMC) oxidoreductases catalyzes the oxidation of β-D-glucose to produce D-gluconolactone and hydrogen peroxide (H_2O_2) utilizing molecular oxygen (Janati-Fard, Housaindokht, & Monhemi, 2016). Glucose oxidase (GOD) is a homodimeric glycoprotein made up of two identical subunits and two non-covalently bound flavin adenine dinucleotides (FAD), which act as an electron carrier during catalysis. The molecular weight of the enzyme ranges from 130 to 175 kDa (Bankar, Bule, Singhal, & Ananthanarayan, 2009). The enzyme was first isolated by Muller (1928), from the mycelia of *Aspergillus niger* and *Penicillium glaucum* and presently, the enzyme is produced on a commercial scale from these two strains of fungi (Rando, Kohring, & Giffhorn, 1997). GOD is reported to be highly specific for glucose and is reported to react with other monosaccharides such as galactose, xylose, and mannose at a very slower rate (Raba & Mottola, 1995).

The enzyme has gained popularity in the food and beverage, chemical, pharmaceutical, clinical, environmental, textile, biotechnology, and other industries during the past few decades. GOD is one of the mostly used enzymes for analytical purposes due to its high specificity, less cost and comparatively high stability (Bankar et al., 2009; Raba & Mottola, 1995). In the food industries, GOD is used as a preservative for stabilizing color and flavor (antioxidant), as an antimicrobial agent, glucose removing agent and as a texture improver in the bakery industry (bread making). GOD is reported to show good antimicrobial activity against food-borne pathogens such as *Salmonella infantis*, *Staphylococcus aureus*, *Clostridium perfringens*, *Bacillus cereus*, *Bacillus subtilis*, *Campylobacter jejuni*, *Yersinia enterocolitica*, *Escherichia coli* and *Listeria monocytogens* (Tiina & Sandholm, 1989; Vartiainen, Rättö, & Paulussen, 2005). There is also a widespread use of GOD for diagnostic purposes to determine blood glucose level and it is a key enzyme used commercially in biosensors for monitoring the glucose level in body fluids (German et al., 2015; Kausaite-Minkstimiene, Glumbokaite, Ramanaviciene, Dauksaite, & Ramanavicius, 2018; Kausaite-Minkstimiene, Simanaityte, Ramanaviciene, Glumbokaite, & Ramanavicius, 2017) due to its high specificity towards glucose compared to other enzyme systems used such as glucose dehydrogenase pyrroloquinone and glucose oxidoreductases (Ferri, Kojima, & Sode, 2011; Schleis, 2007). However, it should be noted that all these commercial applications of GOD mainly focus on the reaction of the enzyme with glucose and there are no reports on the use of the enzyme for detecting other sugars.

Alpha-amylase belonging to the family of endo-amylases is one of the most used industrial enzymes and accounts for about 30% of the world's enzyme production (Sivaramakrishnan, Gangadharan, Nampoothiri, Soccol, & Pandey, 2006). It is widely used in the food,

* Corresponding author.
E-mail address: ruvini@ifs.ac.lk (R. Liyanage).

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fermentation, textile, paper, detergent and sugar industries and with the advancement in the field of biotechnology, the spectrum of α-amylase application has expanded into many other fields, such as clinical, medicinal and analytical chemistry (Bankar et al., 2009). Alpha-amylase catalyzes the initial hydrolysis of starch into maltose, maltotriose, and α-limit dextrins through the cleavage of α-D-(1–4) glycosidic bonds (de Sales, de Souza, Simeoni, Magalhães, & Silveira, 2012). The enzyme neither acts on terminal glucose residues nor on α-1,6-linkages. Thus, there is no production of glucose during this process.

Many methods are being adapted to assess α-amylase activity and most of these methods are cumbersome, less reliable and take a lot of time. Recently, a paper published by our group discusses the development of the GOD method in assessing α-amylase activity (Visvanathan, Jayathilake, & Liyanage, 2016) and to date, it is the first paper published on this regard. In that paper, we have discussed the rationale and justified the interaction of GOD with maltose in detail and developed a simple, rapid, microplate-based method (GOD method) to assess α-amylase inhibitory activity. However, concerns regarding the interaction of GOD with maltose and products of α-amylase has limited the use of the present method in assessing α-amylase activity. The present paper is a continuation of the previous paper (Visvanathan et al., 2016) and the main focus of this study is to determine the applicability of the developed GOD method in assessing α-amylase and α-amylase inhibitory activity and to determine the interaction of GOD with the products of α-amylase.

2. Materials and methods

2.1. Reagents and instruments

α-Amylase (A3306), glucose assay kit (glucose oxidase/peroxidase kit (GOD/POD); BIOLABO), soluble potato starch (33615), sodium phosphate monobasic (71496) and sodium phosphate dibasic (71640) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All the other chemicals used in the study were of analytical grade. Acarbose tablets available in the market were purchased for the study.

2.2. Confirmation of interaction of maltose with GOD

2.2.1. Determination of purity and interaction of maltose standard with GOD by HPLC-RI

Sample preparation: Sugar samples, maltose, and glucose (4 mg/mL) were prepared in milli-Q water and were filtered through 0.45 μm cellulose acetate syringe filters (Greyhound Chromatography and Allied Chemicals, UK). To another set of sugar samples glucose oxidase/peroxidase (POD) reagent was added and was allowed to react for 15 min and the content was freeze dried. Next, the freeze-dried samples were prepared in milli-Q water and were filtered. An aliquot of 1 mL of sugar solutions was placed in vials for the HPLC-RI analysis.

Procedure: The analysis was performed using a HPLC system (AGILENT 1260 INFINITY II LC, Agilent Technologies Inc, 2016) coupled to the refractive index detector (G13624, Agilent Technologies, Waldbronn, Germany) (HPLC-RI). The samples were analyzed using the Agilent's Hi-Plex H column for carbohydrates (300 × 7.7 mm). The injection volume was 20 μL and the flow rate was 0.7 mL/min. The column temperature was maintained at 60 °C and the detector at 55 °C. Sample detection was performed by comparing retention times of the standards.

2.2.2. Determination of products of α-amylase and their interaction with GOD

Sample preparation: α-Amylase was incubated with pre-gelatinized starch for 15 min at room temperature, dipped into ice-water, and was freeze-dried. To another set of digested sample, GOD/POD reagent was added and was allowed to react for 15 min and was freeze-dried. The freeze-dried samples were dissolved in milli-Q water and were filtered through cellulose acetate filters. Finally, the products of α-amylase action were determined through HPLC-RI as described in Section 2.2.1.

2.2.3. Confirming the absence of hydrolysis of maltose to glucose by GOD

For the study, one set of maltose samples were treated with amyloglucosidase (AG) and the absorbance of the AG-treated and untreated samples were compared. In to the wells, 40 μL of maltose (2 mM), 80 μL of PBS and 40 μL of amyloglucosidase (AG) were added. For the AG untreated maltose standard, instead of AG, 40 μL of PBS was added. Finally, 100 μL of glucose kit reagent (GOD/POD) was added and the absorbance was measured for 1 h at 5 min interval.

2.3. Applicability of the method

2.3.1. Sample collection

The following edible plants were chosen to study the α-amylase inhibitory activity using the GOD/POD kit; *Aegle marmelos* (AM), *Hemidesmus indicus* (HI), *Cassia auriculata* (CA), *Scoparia dulcis* (SD), *Phyllanthus emblica* (PE), *Tinospora cordifolia* (TC), *Aerva lanata* (AL), *Sida rhombifolia* (SR), and *Coscinium fenestratum* (CF) were purchased from the Kandy Ayurvedic store and the samples were identified and authenticated by the Herbarium unit of the Peradeniya Botanical Gardens, Peradeniya, Kandy, Sri Lanka.

Ten different types of flours were used to study the hydrolyzing activity of α-amylase. The samples, White raw rice (*Oryza sativa*), Red raw rice (*Oryza sativa*), Corn (*Zea mays*), Chickpea (*Cicer arietinum*), White water lily (*Nymphaea pubescens*), Soy (*Glycine* max), Finger millet (*Eleusine coracana*), and Black gram (*Vigna mungo*) seeds/grains were purchased from the Palwehara seed farm, Dambulla, Sri Lanka. Palmyra (*Borassus flabellifer*) seed-shoots were obtained from a household in Jaffna where the species is grown abundantly. Wheat and Atta flour (*Triticum aestivum*) were purchased from the local market, Sri Lanka. Representative samples were ground in a kitchen grinder mixer and were passed through a 100–150 μm sieve opening to obtain uniform particle size (100–150 μm) flour.

2.3.2. Determination of α-amylase inhibitory activity

Sample preparation: The herbs used for the study were prepared by boiling 20 g of the dried plant material in 200 mL of distilled water and a stock solution of 0.1 g/L was prepared. Acarbose was prepared in phosphate buffer saline (PBS) (0.02 M, pH 6.9). The concentration series used for the study were acarbose (2, 4, 6, 8, 10 μg/mL), *C. auriculata* (0.5, 2, 4, 6, 8, 10 mg/mL), *P. emblica* (0.025, 0.1, 0.2, 0.3, 0.4, 0.5 mg/mL), and others (5, 20, 40, 60, 80, 100 mg/mL).

Procedure: The α-amylase inhibitory activity was determined using the method described by Visvanathan et al. (2016). The assay system comprised the following components in a total volume of 260 μL:40 μL of PBS (0.02 M, pH 6.9), 100 μL of GOD/POD reagent, 40 μL of each, soluble starch (2 g/L), plant extract (inhibitor solution) and the enzyme solution (15 Unit/mL). Briefly, the enzyme solution was mixed with the plant extract and pre-incubated on a hotplate for 10 min at 37 °C. The reaction was started by pipetting the soluble starch solution into each well containing pre-incubated enzyme solution mixed with the plant extract and the content was incubated for another 15 min. Finally, 100 μL of the GOD/POD reagent was added and the absorbance was measured at 505 nm after 15 min using the Thermo Scientific Multiskan GO Microplate Reader.

As a negative control, instead of the sample, 40 μL of PBS was used and the absorbance was measured parallel with samples. For kinetic studies, immediately after the addition of starch, GOD/POD reagent was added and the readings were taken at 1-minute interval for 45 min at 505 nm. The results were expressed in terms of IC_{50} value.

2.3.3. Determination of hydrolyzing rates of starch samples

Hydrolyzing rates of starches were determined using the method described by Visvanathan et al. (2016). First, 4 g/L starch solution was

prepared in PBS (0.02 M, pH 6.9) by heating on a hot plate for 20 min at 100 °C. The enzyme α-amylase (15 Unit/mL) was prepared in PBS. First, 40 μL of starch and 120 μL of PBS were added into microtitre plates. GOD/POD reagent (100 μL) was added and the reaction was started by adding 40 μL of the enzyme into each well and the absorbance was taken up to 45 min at 1 min interval at the wavelength of 505 nm. The linear section of the graph was selected for the calculation of hydrolyzing rate of each starch. Maltose standards of different concentrations subjected to the same treatment were used to quantify the amount of sugar released and the results were expressed as maltose equivalents.

2.4. Statistical analysis

Data were analyzed using the SAS statistical software version 9.1.3 (SAS Institute Inc., Cary, NC). Results were calculated and expressed as mean ± standard deviation (SD) of 3 independent analyses. P values of < 0.05 were considered to be significant. Graphical presentation of data was done using the GraphPad Prism 5 software.

3. Results and discussion

3.1. Confirmation of interaction of maltose with GOD

The present paper discusses the applicability and reliability of using the GOD method in assessing α-amylase activity. The basic principle behind the GOD method is that the products of α-amylase in the presence of O_2 interact with GOD to produce gluconic acid and H_2O_2. The H_2O_2 produced interacts with the kit content (Phenol, 4-aminoantipyrine, and peroxidase) to give rise to a red quinone and its absorbance is measured (Fig. 1). Thus, the amount of color produced is proportional to the amount of sugar reacted with GOD.

Though more than two years have passed, concerns regarding the reliability of the new method, in terms of GOD-maltose interaction, have restricted the widespread use of this method in assessing α-amylase activity. Since glucose oxidase is reported to be highly specific for glucose, many researchers question the interaction of the enzyme with the product of α-amylase (maltose and other dextrins). Wohlfahrt et al. (1999) studied the interaction of other non-glucose sugars with GOD using simulated models. Bonding between glucose, galactose, xylose, and mannose is well documented (Wohlfahrt et al., 1999). However, there's no report regarding the interaction of GOD with maltose. Maltose is a dimer of α-glucose. According to studies done so far, GOD does not show any significant interaction with the alpha form of glucose.

One of the main concerns is the possible contamination of glucose with the maltose standard used in the study, which can also give rise to false positive results. Thus, to eliminate the doubt of any contamination of glucose with maltose and to confirm the interaction of maltose with GOD, the maltose standard used was tested using HPLC-RI. Fig. 2(a) and (b) depicts the HPLC chromatograms for glucose and maltose, respectively. As can be seen from the corresponding chromatogram of maltose (Fig. 2b), there is no glucose in the maltose standard used for the study. The retention time (RT) of maltose was 6.8 while for glucose it was 8.2. Since there is no peak at the RT of 8.2 in the maltose standard (Fig. 2b), it confirms the absence of glucose in the maltose standard. Furthermore, Fig. 2(c) and (d) depicts the corresponding peaks of product formed after reacting glucose and maltose with the GOD/POD reagent. Absence of a peak at RT 6.8 (maltose) in Fig. 2(d) and the formation of a new peak at the RT of 7.2 in maltose confirms the interaction of maltose with GOD.

Fig. 2(e) and (f) shows the hydrolysis products of α-amylase and the resulting product after treating with the GOD/POD reagent, respectively. According to the results, the main product of α-amylase action is maltose. Other than that, two other peaks at the RT of 6.3 and 5.9 were obtained. These may be responsible for the maltodextrins produced during α-amylase action. However, we were not able to clearly identify the dextrins due to lack of dextrin standards of varying chain length. In addition, from Fig. 2(e), it is evident that there is no production of glucose during α-amylase action and this confirms the interaction of maltose with GOD. The digested sample treated with GOD/POD gave a single clear peak at the RT of 7.3 (Fig. 2f) and no other prominent peaks were observed. This confirms the interaction of the products of α-amylase with glucose oxidase.

Though the doubt of interaction of maltose is cleared by the HPLC test results, another concern is the possible hydrolysis of maltose into two glucose units by glucose oxidase. In our previous study, we have proved the interaction of GOD with maltose by comparing the optical density (OD) obtained for amyloglucosidase treated and untreated maltose. However, this work cannot completely eliminate this doubt as we have only taken the result from an end-point assay, where the absorbance readings were taken after 30 min of incubation. Thus to have a clear view, the absorbance readings from both treatments should be taken continuously until a plateau (indicating absence of anymore substrates) is reached. For the confirmation of maltose-GOD interaction, the AG-treated maltose samples should have an approximately two-fold higher OD value than untreated maltose after the reaction reaches a plateau. So to rule out the possibility of hydrolysis of maltose

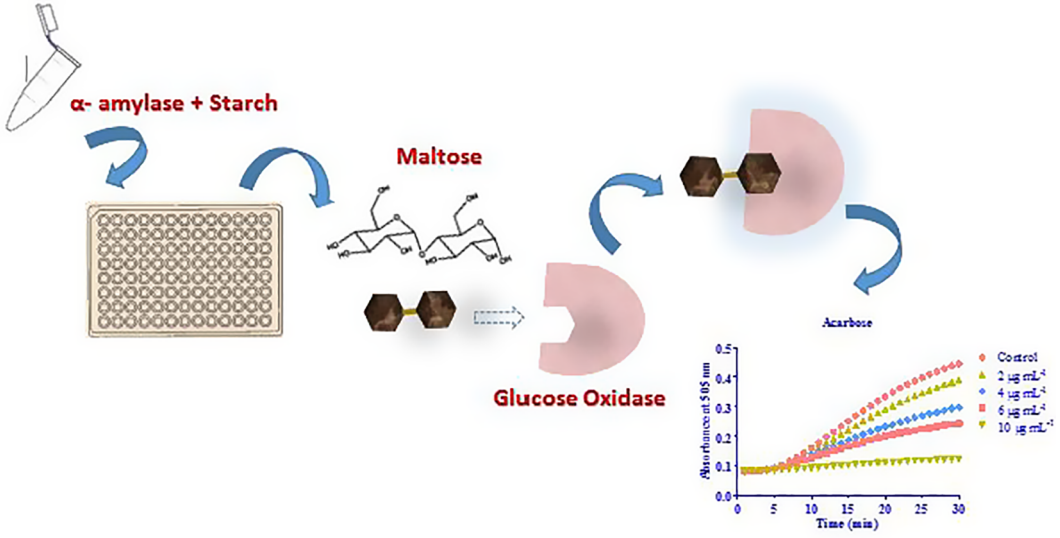

Fig. 1. Schematic representation on the basic principle of the GOD method.

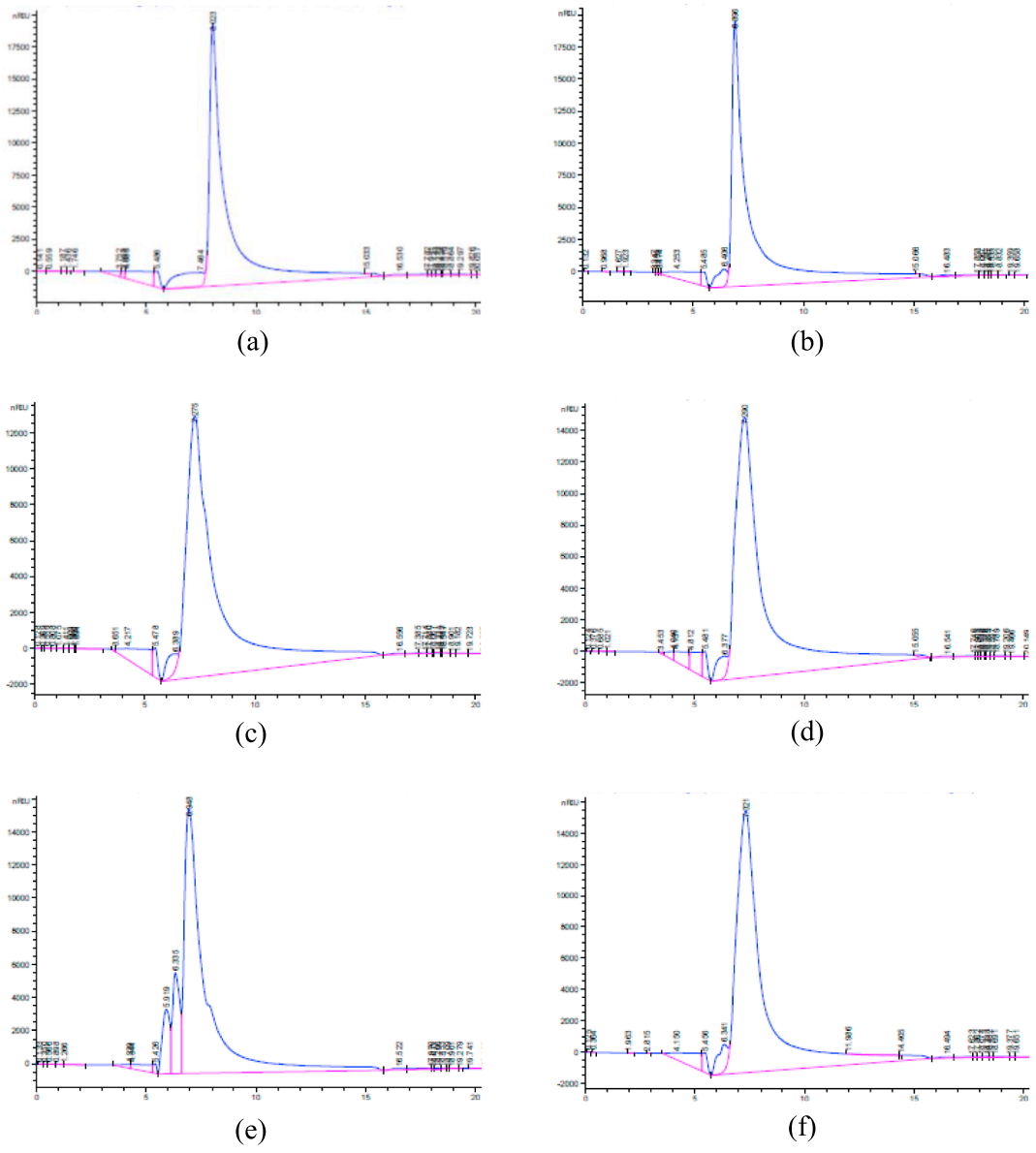

Fig. 2. HPLC chromatograms of (a) glucose, (b) maltose, (c) glucose treated with GOD/POD, (d) maltose treated with GOD/POD, (e) products of α-amylase and (f) GOD/POD treated products of α-amylase.

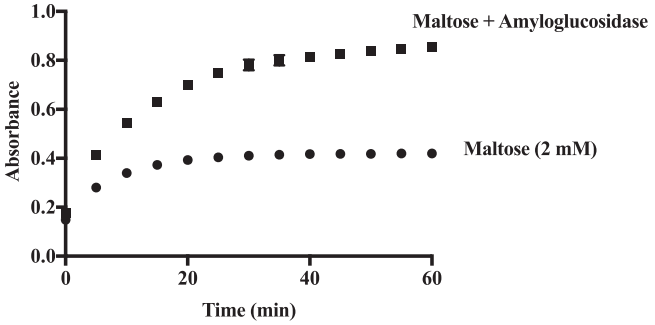

Fig. 3. Reaction rate of amyloglucosidase treated and untreated maltose.

by GOD, the OD values of both the samples were read for one hour at five-minute intervals until a plateau was observed in both the curves. As shown in Fig. 3, the reaction reaches a plateau after some time indicating absence of substrate to take the reaction forward. So if maltose is hydrolysed by GOD, the curve with only maltose should not reach a plateau until the OD value of the AG untreated curve coincides with the OD of the curve with AG. As can be seen from Fig. 3, there's roughly a two-fold difference in the absorbance value of the maltose samples with and without the enzyme AG. Maltose has one reducing end while hydrolysis of maltose into glucose produces two reducing ends. Thus, the increases in absorbance of AG treated samples are due to hydrolysis of maltose into its monomer glucose. This observation further confirms the interaction of maltose with GOD and rules out the doubt of hydrolysis of maltose into glucose by GOD.

3.2. Practical applicability

3.2.1. Amylase inhibitory activity

The study on α-amylase inhibitory activity of plant compounds is a widely studied area which may be relevant to the treatment of Type 2 Diabetes Mellitus (T2DM) (Jarald, Joshi, & Jain, 2008; Patel, Prasad, Kumar, & Hemalatha, 2012). Medicinal plants are widely used in the *Ayurvedic* system to treat T2DM (Jarald et al., 2008). Over 1200 species of plants representing 725 genera and 183 families are been listed as

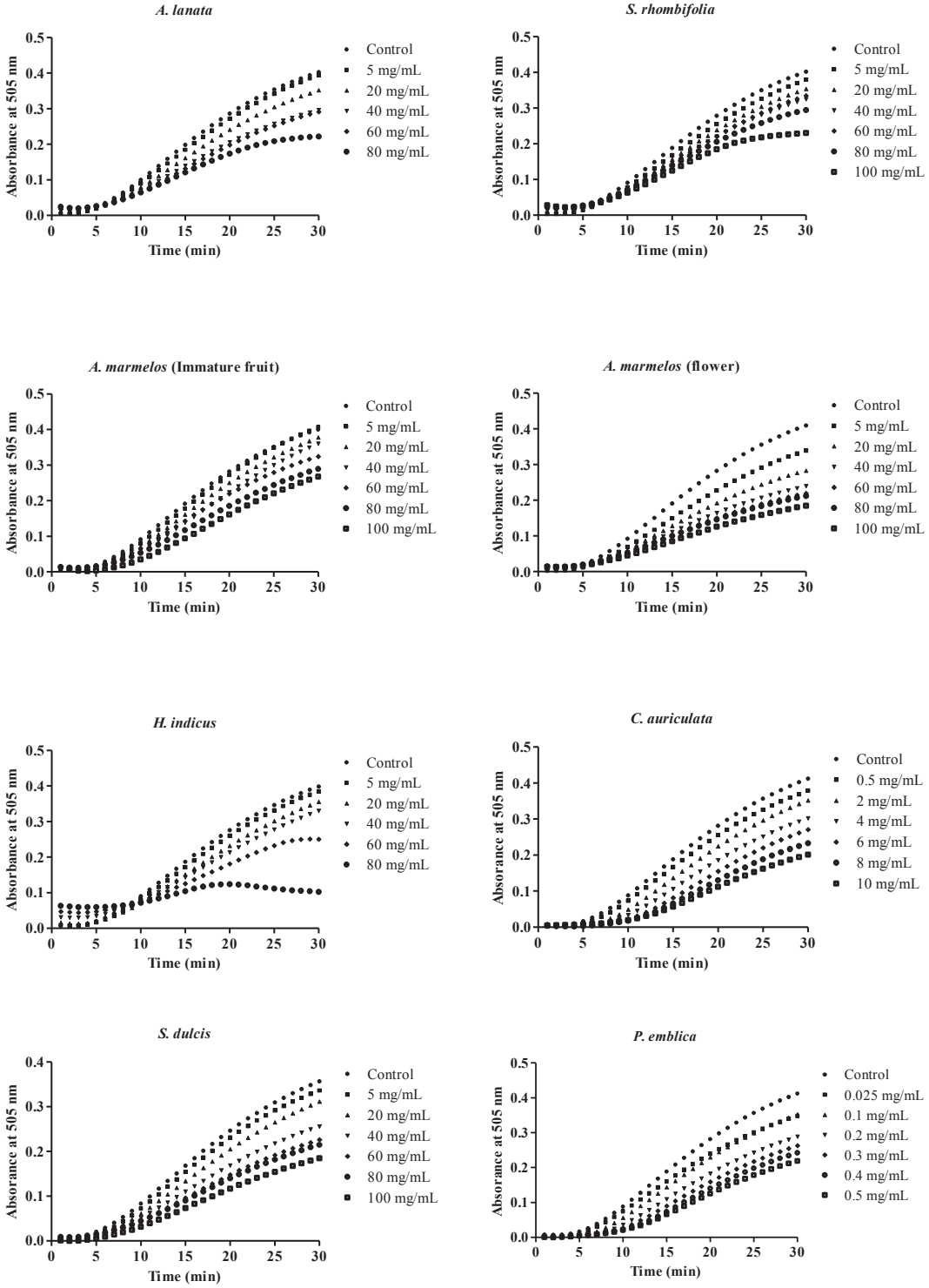

Fig. 4. Representative kinetic curves of α-amylase inhibitory activity of 10 medicinal plants and the positive control acarbose.

anti-diabetic plants in the NAPRALERT database (Jarald et al., 2008). In the present study, the anti-amylase activity was studied using natural extracts of ten herbal plants widely used in the *Ayurvedic* system to treat diabetes mellitus. PE, CF, HI and AM (bark and root) are widely used in the Sri Lankan *Siddha* medicinal system to treat DM (Sathasivampillai, Rajamanoharan, Munday, & Heinrich, 2017). Except for SR and HI, all the other plants used in this study are scientifically validated as anti-diabetic plants and PE, CA, TC, AM, and SD are widely used in *Ayurvedic* drug formulations available in the market for the treatment of diabetes (Jarald et al., 2008).

The inhibition kinetic curves and the IC_{50} values of the natural extracts are presented in Fig. 4 and Table 1 respectively. It can be clearly seen from Fig. 4 that in all the tested samples the absorbance value decreases with an increase in the extract concentration indicating reduced enzyme activity. For each sample concentration, a blank reading was carried out to eliminate the effect of sample color and free sugars present in the sample. IC_{50} value is the amount of compound required to inhibit the enzyme activity by 50%. Thus, lower the IC_{50} value higher is the inhibitory activity. All the tested extracts showed potent α-amylase inhibitory activity. A significant difference ($P < 0.05$) was observed between the α-amylase inhibitory activities

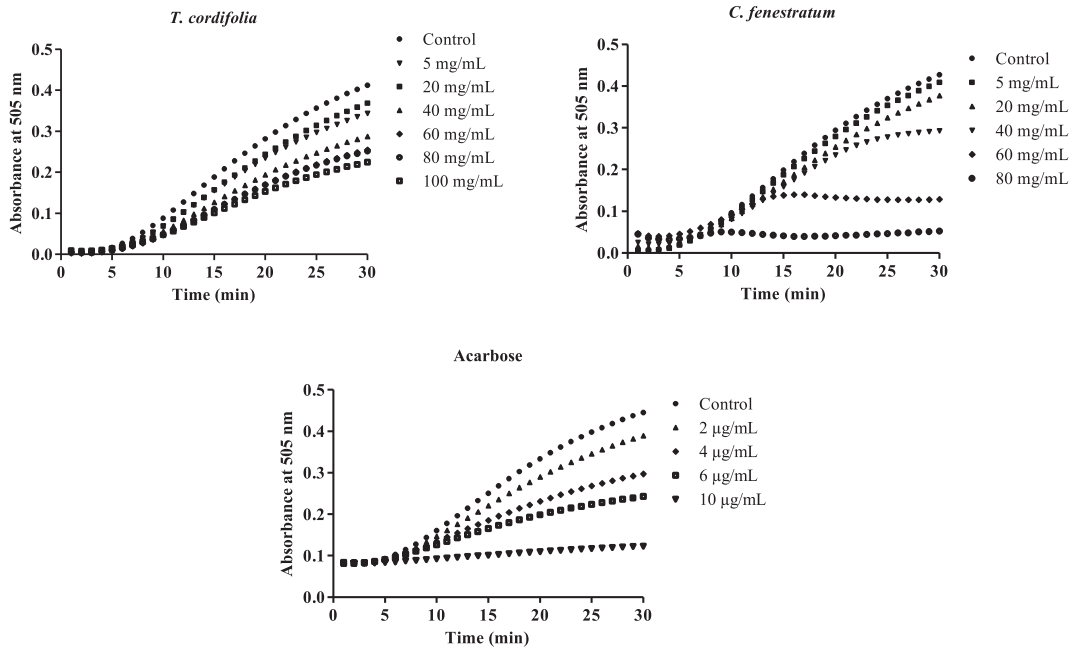

Fig. 4. (*continued*)

Table 1
α-Amylase inhibitory activity of herbs.

Plant type	Amylase inhibitory activity IC_{50} (mg/mL)
A. lanata	67.39 ± 0.07[d]
S. rhombifolia	104.37 ± 4.70[b]
A. marmelos (Immature fruit)	109.41 ± 3.21[a]
A. marmelos (Flower)	43.11 ± 0.68[f]
H. indicus	52.30 ± 1.22[e]
C. auriculata	7.16 ± 0.04[g]
S. dulcis	64.88 ± 2.46[d]
P. emblica	0.37 ± 0.002[h]
T. cordifolia	74.73 ± 1.05[c]
C. fenestratum	41.60 ± 1.07[f]

Data are presented as mean ± standard deviation (n = 3). Different letters within a column indicate significant differences ($P < 0.05$).

of the extracts, which ranged from 0.37 ± 0.002 to 109.41 ± 3.21 mg/mL. According to the results (Table 1), *P. emblica* extract showed significantly higher inhibition of α-amylase while least activity was observed in the *A. marmelos* fruit extract. Several papers have reported the effectiveness of *P. emblica* extract in controlling hyperglycemia (Duraiswamy, Shanmugasundaram, Sasikumar, Cherian, & Cherian, 2016; Poongunran, Perera, Fernando, Jayasinghe, & Sivakanesan, 2015). In a study done by Poongunran et al. (2015), the methanolic extract of PE showed potent α-amylase inhibitory activity with an IC_{50} value of 0.397 mg/mL and the activity of PE was significantly higher than TC which perfectly correlates with our findings. However, according to the results, none of the tested extracts were as effective as acarbose (IC_{50} = 5.50 ± 0.07 µg/mL) in inhibiting the enzyme.

3.2.2. Hydrolyzing rates of starches

Hydrolyzing rates of the starches for α-amylase ranged from 0.23 ± 0.01 to 12.52 ± 0.10 µM maltose/min (Fig. 5a, b). The highest maltose releasing rates were observed in wheat flour followed by atta (10.89 ± 0.47 µM maltose/min), white raw rice (10.44 ± 0.42 µM maltose/min) and finger millet (9.21 ± 0.33 µM maltose/min). The lowest rates were observed in soy (0.23 ± 0.022 µM maltose/min) followed by white lily seeds (1.87 ± 0.09 µM maltose/min), black gram (3.65 ± 0.26 µM maltose/min) and chickpea (4.73 ± 0.13 µM maltose/min). There was no significant difference in the hydrolyzing rates of red raw rice (8.54 ± 0.08 µM maltose/min) and palmyra (8.12 ± 0.26 µM maltose/min) (Fig. 5b). As seen in Fig. 5(a), soy bean samples showed no significant release of maltose and the hydrolyzing curve for soy was flat. Mature soy beans are reported to contain little or no starch. Wilson, Birmingham, Moon, and Snyder (1978) studied the starch content in 10 varieties of soy beans and reported the starch content to range between 0.19 ± 0.01 and 0.91 ± 0.08%. In addition, heating is reported to disrupt

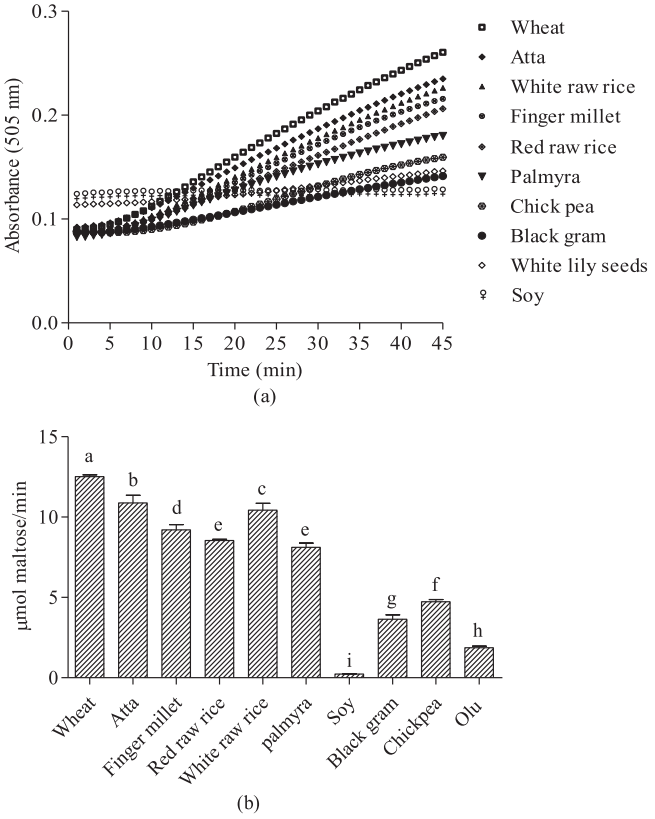

Fig. 5. (a) Kinetic curves for hydrolyzing rate and (b) maltose releasing rate of starches.

the starch granules in soy beans (Wilson et al., 1978). It should be noted that while preparing the starch solution for analysis, it was gelatinized at 100 °C for 20 min. Thus, the flat curve observed may be as a result of the low content/absence of starch in soy beans.

The first stage in the digestion of starch is catalyzed by α-amylase which results in the production of maltose, maltotriose and limit dextrins as the main products (Butterworth, Warren, & Ellis, 2011). Considerable differences have been reported in the postprandial blood glucose level after ingestion of identical amounts of foods prepared from different origins (Pirasath, 2015; Widanagamage, Ekanayake, & Welihinda, 2009). Such differences are observed due to the differences in the rate and extent of starch digestion in the gastrointestinal tract, which is attributed to the inherent differences in the structure of starch granules and other components present in different botanical sources. Several factors are reported to affect starch digestibility including, dietary fiber content, protein content, lipid content, physical and chemical characteristics of the starch granule, particle size, cooking conditions, and the possible presence of a natural α-amylase inhibitor in the sample (O'Dea, Snow, & Nestel, 1981; Snow & O'Dea, 1981; Widanagamage et al., 2009). In many studies, wheat is reported to hydrolyze at a higher rate than other starches due to its 'A' type starch granules and the presence of surface pores that aid in the attachment of the enzyme. Cereal starches (wheat, atta, rice, finger millet) have 'A' type crystallinity which acts as a more favorable substrate for α-amylase than tuber (Palmyra) starches possessing 'B' polymorph and legume starches (chickpea, black gram, soy) having 'C' pattern of crystallinity (Butterworth et al., 2011). As to support our results, several other studies have showed that the glycemic index (GI) value of red rice to be less than the glycemic value of white rice which was attributed to its high fiber content that act as a physical barrier to limit access of the hydrolytic enzymes to the starch (O'Dea et al., 1981; Pirasath, 2015; Snow & O'Dea, 1981). In a study done by Pirasath (2015), wheat bread had a higher glycemic value than red rice and white rice. Food items, such as 'Idly' and 'Thosai', prepared using a mixture of rice flour and black gram flour had a lower GI value than food prepared from solely wheat flour and rice flour. In the same study, boiled chickpea was shown to have a very low GI value and was categorized under the low GI food group. As the *in vitro* hydrolyzing rate of starch by α-amylase is reported to perfectly correlate with the postprandial glucose response in humans after a meal (O'Dea et al., 1981), the GOD method can be useful in assessing the hydrolyzing rates of starches by α-amylase which indirectly depicts the metabolic responses to a particular starchy diet.

The novel GOD method has several advantages over the conventional α-amylase methods, such as the dinitrosalicylic acid (DNSA), Nelson-Somogyi, iodometric and turbidimetric methods. The most striking advantage of the GOD method is that it can be directly done on microtiter plates, which reduces labor, sample and reagent requirement and consumes less time. The GOD method only involves a few steps and 3 to 4 samples can be assayed at once relatively within 40 min (Visvanathan et al., 2016). On the other hand, the DNSA and the Nelson-Somogyi methods are highly labor intensive and consumes a lot of time due to the involvement of several steps including the heating step, which is required for color development (Saqib & Whitney, 2011; Shao & Lin, 2018). The boiling step, addition of cold water and transfer of the reaction mixture from the test tubes to cuvettes/microplates are very time consuming, especially when handling of a large number of samples is considered (Gonçalves, Rodriguez-Jasso, Gomes, Teixeira, & Belo, 2010; Shao & Lin, 2018). Unlike some of the conventional methods, the GOD method does not involve any complex hazardous chemical preparation. Furthermore, the GOD method shows approximately 4 times higher sensitivity towards maltose, the main product of α-amylase, than the DNSA method (Visvanathan et al., 2016). Though the iodometric and turbidimetric methods are modified to the microtitre plate, there are some significant shortcomings, which have limited the use of these two methods. In the iodometric method, since the starch-iodine complex forms a deep blue color, measurement of a relatively small change in absorbance is quite challenging. On the other hand, in the turbidimetric method, sedimentation of the substrate and getting a homogenous mixture into each well makes the method more difficult and less reliable. Although fast and relatively simple, preparation of stable and reproducible starch solutions has limited the use of these two methods.

4. Conclusion

The present work aimed at studying the applicability and reliability of GOD in assessing α-amylase activity. The interaction of maltose with GOD has been proved in every possible way and there is no doubt in the interaction of maltose with GOD and other products of α-amylase. Furthermore, GOD was successfully used to determine α-amylase and α-amylase inhibitory activity of the tested samples. In summary, it can be said that the GOD method is an accurate and reliable method for quantifying α-amylase activity and there is no doubt on the interaction of GOD with the products of α-amylase. The method is less complicated and is also well suited for kinetic studies and this particular assay can serve as a platform to screen large populations of α-amylase inhibitors easily within a less time.

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Conflict of interest

The authors declare that there is no conflict of interest.

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