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Critical review on conventional spectroscopic α-amylase activity detection methods: merits, demerits, and future prospects

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Abstract

α-Amylase is an endoenzyme that catalyses the hydrolysis of internal α-l,4 glycosidic linkages in polysaccharides to produce maltose, maltotriose, and α-limit dextrins. It is widely used in the laboratorial and industrial workflow for several applications. There are several methods utilizing different techniques and substrates to assess α-amylase activity, among which the spectroscopic methods have found widespread applicability due to their ease of use and cost-effectiveness. Depending upon the reaction principle, these assays are classified into four groups: reducing sugar, enzymatic, chromogenic, and amyloclastic methods. Despite the presence of numerous methods, there is no general reliable method to assess α-amylase activity. Each method is shown to have its own merits and demerits. Many improvements have been made to make the available methods more accurate, reliable, and easy. This communication briefly discusses the basic reaction mechanisms and critically reviews the advantages and shortcomings associated with each method. Further recommendations are made for future development.

Keywords: α-amylase; detection methods; reaction mechanism; advantages; shortcomings; future prospects

INTRODUCTION

The amylases (α-amylases, β-amylases, and glucoamylases) are one of the most important families of enzymes that have found wide applicability in the field of biotechnology.[1] Among these, α-amylases (E.C. 3.2.1.1.) are widely used industrially, accounting to about 25–30% of the world's total enzyme production and are the first enzyme to be prepared on a commercial scale.[2,3] α-Amylases are endo-acting enzymes that catalyse the initial hydrolysis of starch into shorter oligosaccharides through the cleavage of internal α-D-1,4-glycosidic bonds with the retention of α-anomeric configuration in the products.[4–6] They act at random locations of the starch molecule, yielding oligosaccharides with varying chain length, which constitute a mixture of maltose, maltotriose, and branched oligosaccharides of six to eight glucose units that contain both α-1,4 and α-1,6 linkages (Fig. 1). Neither terminal glucose residues nor α-1,6-linkages can be cleaved by α-amylase.[7] Thus, theoretically, there is no production of glucose in this process.

α-Amylase activity is measured for several reasons industrially and diagnostically. Industrially, α-amylase monitoring is critical in maintaining the quality of final products, and clinically it is mainly used to identify some disease conditions associated with the pancreas and salivary glands. Naturally present α-amylases in foodstuffs, such as honey, raw fruits and vegetables, cereal grains, and fermented foods, degrade the quality of some processed food items.[8] Both high and low levels of α-amylase are reported to have a huge impact on the final quality of bread, thus making it essential to measure the activity of the enzyme to maintain the final quality of food products.[9,10] Furthermore, α-amylase is used in warp sizing of textiles, as an additive in detergents to remove starchy soils, and in the paper industry to reduce the viscosity of starch to ensure appropriate coating of paper.[2,11] Clinically, α-amylase activity in serum and urine is widely measured to diagnose acute pancreatitis, and it is also used as a digestive aid.[12] Thus, owing to its importance in the field of diagnostics and food production and other commercial applications, it is important that assays that measure α-amylase activity be highly objective and selective.

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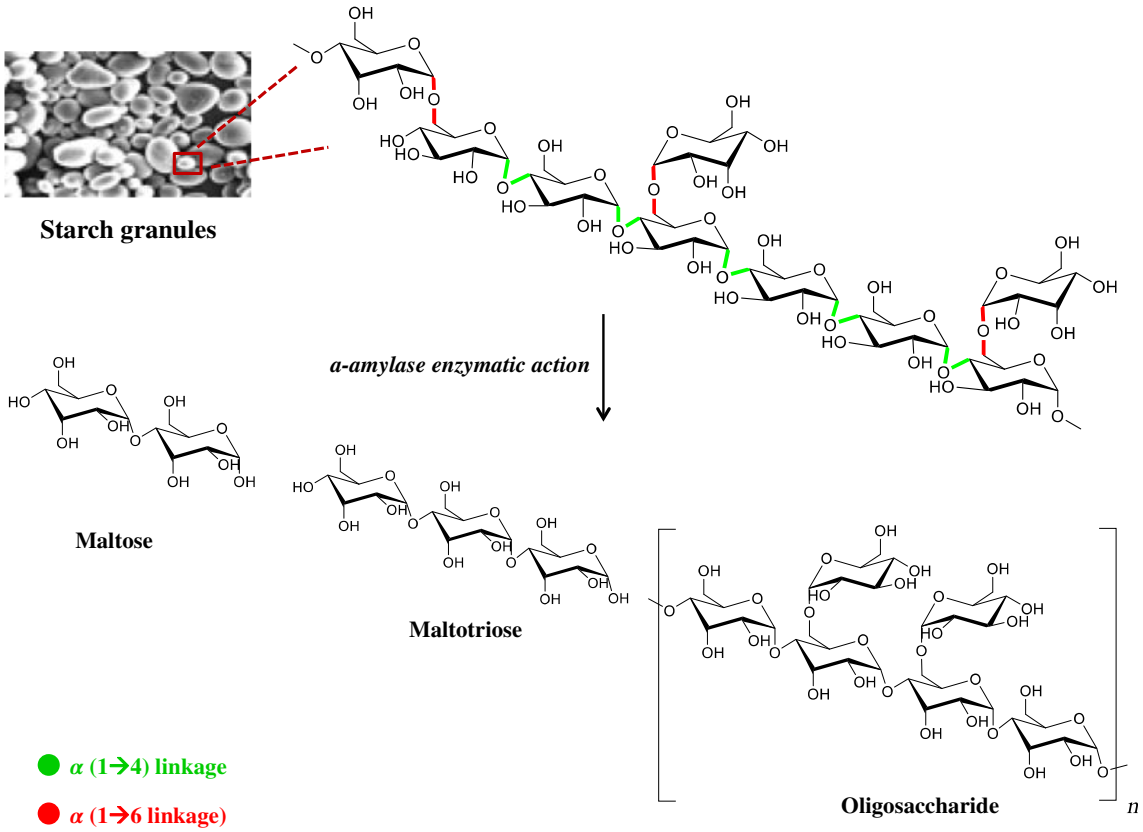

Figure 1. Graphical representation of α-amylase action on starch.

There are several methods reported in the literature to measure α-amylase activity. α-Amylase is probably the enzyme with the most published analytical procedures to assess its catalytic activity. A PubMed search itself results in more than 1500 papers for α-amylase activity detection methods, which indirectly reflects the difficulties in determining the amylolytic activity. Since the majority of the reported methods are cumbersome, less reliable, and take a lot of time to perform, most of these papers are based on modifying the available methods to increase accuracy and ease of use. In the literature, several papers describing different kinds of analytical techniques to determine α-amylase activity can be found.[13–19] For example, techniques such as spectrometry,[16,20] fluorometry,[8] amperometry,[18] electrophoresis, isoelectric focusing, chromatography, and immunological methods[19] have been adapted to detect α-amylase activity. In addition, several methods have emerged that use different substrates, including starch, amylose, amylopectin, and some chemically modified derivatives of polymers and maltooligosaccharides of varying chain length linked to a chromophore, such as 4-nitrophenyl or 2-chloro-4-nitrophenyl.[21] Though several techniques are reported, the most common technique employed widely in the clinical and commercial field is spectrophotometry, owing to its ease of use and cost-effectiveness. Thus, this review will focus on discussing the most commonly used spectroscopic α-amylase activity detection methods.

IMPORTANCE OF ASSESSING α-AMYLASE ACTIVITY

α-Amylase is widely used in the food and beverage, fermentation, textile, paper, detergent, fuel, and sugar industries, and with the advancements in the field of biotechnology, the spectrum of α-amylase application has expanded into many other fields, such as clinical, medicinal, and analytical chemistry.[22] The quality of starch-containing foods is significantly influenced by the action of naturally present α-amylase in foods, which can hydrolyse starch and eventually result in viscosity loss. Starch is mainly used as a thickening agent in many food preparations, such as sauces, soups, and canned stews and curries.[8] The presence of even a small amount of α-amylase can hydrolyse the starch and result in significant viscosity loss and eventually degrade the quality of the final product.[8] In egg processing, α-amylase activity is measured to ensure the efficiency of the pasteurization process.[23] Thus, for quality control purposes, it is necessary to measure residual α-amylase activity in these food materials to ensure the quality of the final product. Furthermore, α-amylase present in flour samples has a profound effect on the quality of bakery products, especially of bread, where it plays an important role in improving the loaf volume and shelf life.[9,10] The presence of high levels of α-amylase in flours reduces bread volume and breadcrumb strength and increases the levels of starch degradation products, resulting in a sticky crumb.[10,24] On the other hand, very low levels of α-amylase are also associated with reduced loaf volume, poor quality crumb, and reduced shelf life of bread.[24] Thus, α-amylase activity monitoring becomes indispensable in maintaining the quality of bakery products.

In paper processing, the coating process determines the quality of paper; that is, proper coating of starch is essential in producing smooth and strong paper with improved writing quality. In this process, α-amylase is used to reduce the viscosity of natural starch, which is otherwise too high for paper sizing. Too high

and too low α-amylase activity is reported to have a huge impact on the overall quality of paper.[2,11] In other industries, such as the textile industry, α-amylase is extensively used to remove starch applied for warping of fabrics;[25] and in detergent formulations, α-amylase is used as a principal ingredient to remove starch-based soil from cloths.[2,11] α-Amylase is used in wastewater treatment plants to hydrolyse starch-based wastes and to produce value-added products such as maltose, high-fructose corn syrup, oligosaccharides mixtures, maltotetraose syrups, high-molecular-weight-branched dextrins, and bio-alcohols.[26] Moreover, α-amylase is an essential ingredient in the production of gypsum boards required for dry wall construction.[11] In all these processes, α-amylase activity is monitored to ensure the quality of the products. Thus, it is evident that all these industrial applications depend on the reaction rate of α-amylase, making it essential to monitor the activity of α-amylase as a measure of quality control.

Other than the industrial applications, α-amylase activity is also assessed for research purposes. For example, α-amylase activity is measured to study the inhibitory effect of naturally present phytochemicals on α-amylase, which is believed to be one of the main remedies in the treatment of diabetes mellitus.[27–29] In humans, α-amylase is predominantly produced in the parotid glands and pancreas. As the intestinal epithelium is only capable of absorbing monosaccharides in humans, before being absorbed as glucose in the small intestine, starch is successively hydrolysed by salivary and pancreatic α-amylases in the mouth and small intestine, respectively, to its respective smaller oligomers.[30,31] The resultant product of α-amylase action is further hydrolysed into glucose by the combined action of two intestinal brush border enzymes: maltase-glucoamylase and sucrase-isomaltase.[31,32] Though other amylolytic enzymes participate in the process of starch breakdown, the contribution of α-amylase is the most important for the initiation of this process. Therefore, α-amylase is one of the key enzymes of great concern to medical practitioners and researchers in controlling the pandemic of diabetes mellitus.[33] Compounds present in plants are reported to act as inhibitors of α-amylase, and several studies have been conducted to examine the anti-amylase activity of these plant extracts.[34,35] To date, about 800 plant species have been reported to exert anti-diabetic activity.[30] Compounds such as alkaloids, glycosides, flavonoids, carotenoids, polysaccharides, hypoglycans, peptidoglycans, guanidine, steroids, glycopeptides, and terpenoids are reported to be responsible for the exerted bioactivity against hyperglycaemia.[27,29] Thus, studying the α-amylase inhibitory effect of these compounds is considered as an effective therapy in controlling the development of diabetes.

In addition to this, in the clinical set-up, α-amylase activity is measured widely in serum and urine to detect abnormality in the pancreas and salivary glands and in some special cases in other body fluids, such as the peritoneal fluid.[12,36,37] α-Amylase in the human body is present in two isoforms: P-type and S-type.[7,12] P-type α-amylases are synthesized in the pancreatic acinar cells and are secreted into the intestinal tract via the pancreatic duct system. S-type α-amylase is found in secretions of saliva, testes, ovaries, Fallopian tubes, Mullerian ducts, striated muscle, lungs, and adipose tissue, as well as in semen, colostrum, tears, and milk.[12] The major production site of S-type α-amylase is the salivary glands, where it initiates the hydrolysis of starch while food is in the mouth and oesophagus, and its action is terminated by acid in the stomach. About 20% of α-amylase in the plasma is excreted by the kidneys,[37] and the major catalytic site of the remainder is the liver.[12] The normal range for serum α-amylase activity is approximately 36–128 U L^{-1}.[36] A higher than normal α-amylase activity may indicate the presence of several disease conditions, such as parotitis, pancreatitis, perforated peptic ulcer, pancreatic cancer, torsion of an ovarian cyst, strangulated ileus, macroamylasaemia, mumps, renal failure, and other malignant conditions, such as breast, colon, lung, and ovarian cancers.[12,36] Thus, measurement of serum α-amylase activity is considered as a useful tool in screening for patients with abnormalities associated with high α-amylase activity, and there are several methods reported to measure the activity of α-amylase during pathological conditions.[38–40]

METHODS USED TO ASSESS α-AMYLASE ACTIVITY

There are several spectroscopic methods in use to determine α-amylase activity and all these methods are based on measuring one of the following properties of the substrate: (i) decrease of viscosity of a starch solution, (ii) decrease of turbidity of a starch suspension, (iii) decrease of intensity of a starch-iodine reaction and (iv) increase of reducing power.[28] The former three methods measure the amount of substrate (starch) remaining after an enzymatic reaction while the latter measures the amount of product formed (reducing sugars). However, methods based on assessing the amount of product formed are more in use due to accuracy and easiness. The following section will discuss on the mostly adapted spectroscopic methods in use to assess α-amylase activity and a brief overview on the discussed methods is presented in Table 1.

Reducing sugar methods

A number of reducing methods are used to detect α-amylase activity, including the 3,5-dinitrosalicylic acid (DNSA),[13] Nelson–Somogyi (NS),[41] copper–bicinchoninate (CuBic),[42] and the Park and Johnson (PJ)[43] method. All these methods are based on measuring the oxidizing ability of reducing sugars. Sugar molecules act as reducing agents as long as they contain an aldehyde group and exist in an open-chain structure. Monomeric sugars exist in aqueous solution in equilibrium between their open-chain and ring structures but only the open structures are responsible for their reducing activity. During dimer formation, the aldehyde group of one of the sugars is buried in the glycosyl bond, making it incapable of acting as a reducing agent anymore.[50]

Among the reducing sugar methods, the DNSA method is the most commonly used protocol for measuring α-amylase activity. The DNSA method was first described to determine reducing sugars in normal and diabetic urine.[13,51] The method was later optimized by Noelting and Bernfeld[52] to measure reducing sugars released by the action of α-amylase and has also been widely used to measure other carbohydrase activities.[53–55] The common steps involved in the DNSA method can be summarized as follows. Step 1: addition of enzyme and starch into test tubes; step 2: addition of DNSA reagent; step 3: heating; step 4: cooling; step 5: addition of distilled water; step 6: transfer of the reaction mixture into a cuvette or microtitre plate; and finally, step 7: measurement of absorbance at 540 nm. Though this method is the most cited method in the literature to assess α-amylase activity, there are some significant drawbacks.

The basic principle behind the DNSA method lies in the reaction of the aromatic DNSA with the carbonyl end of the reducing sugars to yield the deep-orange-coloured 3-amino-5-nitrosalicylic acid (ANS), which absorbs light strongly at 540 nm:

Table 1. Brief overview on α-amylase detection methods

	Reducing sugar methods	Enzymatic method (GOD/POD)	Chromogenic method	I_2–starch method	Turbidimetric method
Assay principle	Based on the oxidizing ability of sugars	Based on the interaction of glucose/products of α-amylase with GOD	Interaction of the substrate with α-amylase and release of the chromophore upon hydrolysis by a α-glucosidase	Based on the interaction of I_2 and I_3^- with the amylose helix structure	Based on the reduction of turbidity of a starch solution due to breakdown of starch
Examples	DNSA, NS, PJ, CuBic	GOD/POD method	Phadebas, Ceralpha	I_2–starch method	Turbidimetric method
Reagent preparation	Involves preparation of several chemicals	Only starch and amylase	Amylase, glucosidase, and the substrate	Involves preparation of several chemicals	Only starch and α-amylase
Substrate	Starch	Starch	Maltooligosaccharides (G3–G10) linked to a chromophore, azurine cross-linked amylose	Starch, amylose	Starch
Microplate-based method	Available, but requires heat-resistant plates	Available	Available	Available	Available
Steps involved					
Reagent addition	✓	✓	✓	✓	✓
Heating	✓	—	—	—	—
Cooling	✓	—	—	—	—
Addition of colour reagent	✓	—	—	—	—
Standing time for colour development (min)	5–20	5–15	10–15	—	—
Dilution	✓	—	—	Sometimes required	—
Total assay steps	6–8	3–4	4	6	2–3
Minimum detection limit	DNSA: 2.92 mmol L^{-1}* NS: 0.03 mmol L^{-1}** CuBic: 5 µmol L^{-1}* PJ: 5 µmol L^{-1}**	0.73 mmol L^{-1}*	1.43 U L^{-1}***	N/A	1 U L^{-1}***
Absorbance reading (nm)	540–620	500–505	405	550–700	600–660 nm
References	13,41–45	33,46	39	47,48	16,49

Minimum detection limit in terms of *maltose, **glucose, and ***amylase enzyme activity.
N/A, not available.

$$\text{Sugar} + \text{DNSA} \rightarrow \text{ANS} + \text{Sugar acid} \quad (1)$$

(yellow) (deep orange)

In other words, while the DNSA is reduced to ANS, the functional groups (aldehyde/ketone) of the sugars are simultaneously oxidized to their respective carboxylic acids. Thus, theoretically, the amount of ANS produced should be directly proportional to the number of functional groups oxidized in the sugar. However, several studies have reported different sugars to yield different colour intensities, indicating non-equivalence between the amount of sugar reacted and ANS produced.[44,50,56,57] This problem was first reported in a study done by Robyt and Whelan,[56] where the use of the DNSA assay for measuring α-amylase activity showed a non-linear relationship between the amount of colour produced and the amount of actual number of hemiacetal reducing groups present in the sample. The values obtained showed a positive relationship with the size of the maltodextrin chain, where the reducing value increased with an increase in the size of the dextrin chain. The chain-length dependency was believed to be due to overoxidation of the maltodextrins caused by an alkaline erosion of the maltodextrin chain, which significantly affected the quantitative reliability of the α-amylase assay. The same was observed in a study done by Saqib and Whitney,[50] where disaccharides gave a greater colour than the monosaccharides. However, galactose, an aldose same as glucose showed significantly different value than glucose with DNSA suggesting the chemistry of the test to be appreciably more complicated than just the production of ANS from DNSA. However, galactose, an aldose, as with glucose, showed a significantly different value from glucose with DNSA, suggesting the chemistry of the test to be appreciably more complicated than just the production of ANS from DNSA.[44,50] Thus, the use of the DNSA method for the determination of α-amylase

gives only a qualitative estimate of reducing groups and it cannot be recommended to measure specific activity of α-amylase or any other enzymes that yield a mixture of sugars. However, the DNSA method can be useful for defined product assays, such as in the measurement of β-amylase on amylopectin, where maltose is formed exclusively.[45] Furthermore, the presence of some amino acids[58] and polyphenols[59] has been reported to interfere with the assay. The interference from polyphenols was corrected by passing the reaction mixture through a solid-phase extraction unit.[59]

Some of the other main disadvantages of the DNSA method are the requirement of high volumes of sample and reagents, the involvement of several steps, including the heating step, high time consumption, and being labour intensive. 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.[60] At the time of its discovery, the DNSA method was preferred among other reducing sugar methods as per its great convenience. However, with the advancement of chemistry and analytical techniques, at present, the DNSA method cannot be considered as a convenient method. In addition to reducing time, chemicals, and labour, assay systems functioning on a microlevel facilitate rapid screening of a large number of samples. Two papers have discussed the adaptation of the DNSA method to microtitre plates.[60,61] However, the requirement of a special type of heat-resistant microtitre plate has limited the use of this modified method.

The next widely used method for the determination of α-amylase activity is the NS method,[41,62] which is based on the copper reduction principle. In this assay, the sugars are heated along with copper tartrate under alkaline conditions to form cuprous oxide, then allowed to react with arsenomolybdic acid to yield molybdenum blue, which absorbs light at 620 nm:[58]

$$\begin{gathered}\text{Sugar} + \text{Cupric ion}\left(Cu^{2+}\right) \rightarrow \text{Cuprous ion}\left(Cu^{+}\right) + \text{Sugar acid} \\ \text{Arsenomolybdate complex} + Cu^{+} \rightarrow \text{Molybdenum blue} + Cu^{2+} \\ \text{(colourless)} \qquad\qquad \text{(blue)}\end{gathered} \tag{2}$$

The steps involved in the NS method are as follows. Step 1: addition of reagents into test tubes; step 2: heating; step 3: cooling; step 4: addition of colour reagent; step 5: standing for 15 min; step 6: addition of distilled water (dilution); step 7: transfer of the reaction mixture into a cuvette or microtitre plate; and finally, step 8: measurement of absorbance at 620 nm.

Unlike the DNSA method, the NS method was found to give more accurate results in measuring reducing sugars. This particular method was reported to generate identical reducing values for equimolar quantity reduction of maltodextrins. In other words, the measurement of the apparent maltose produced in an α-amylase reaction was directly proportional to the specific activity of the enzyme present.[57] The overoxidation problem encountered in the DNSA method was not observed in the NS method in a study done by Robyt and Whelan,[56] where the reducing value of maltose, maltooligosaccharides, and maltodextrins showed an apparent stoichiometric relationship with the available hemiacetal reducing groups. In addition, the NS method is reported to be ten times more sensitive than the DNSA method.[55,56] However, care should be taken to prevent reoxidation of Cu(I) to Cu(II) during the heating and cooling period before arsenomolybdate reagent addition, as back-oxidation has a high possibility of reoxidation.[63]

Despite its high accuracy and sensitivity, the NS method has received less acceptance/popularity in terms of usage compared to the DNSA method. The main reason for this is the cumbersome steps involved in the NS method. The DNSA assay is most popular because it is comparatively simple and fast. The method only involves the steps of mixing and heating the sugar solutions with the DNSA reagent for 5 min, followed by cooling and absorbance reading.[13,51] On the other hand, the NS method has long heating step (20 min), followed by the addition of colouring agent and dilution. However, though the NS copper method is less convenient than the DNSA method, it has been reported to be more reliable and more sensitive in terms of accuracy and reliability.[57,64]

Both the NS and the DNSA methods require a large number of reaction tubes and transfer of the final solution to cuvettes/microtitre plate for absorbance reading. In addition, handling a large number of tubes during the heating process (in a water bath) limits the analytical capacity (number of samples that can be assayed at a time) and efficiency of the methods. To overcome this problem, Shao and Lin[45] recently developed a more convenient microplate-based NS method for the determination of reducing sugars that has significantly reduced the assay time (25 samples can be assayed within approximately 1 h) and reagent usage and has also increased the sensitivity and accuracy of the assay. However, this method also requires a heat-resistant polypropylene microplate to withstand the heat to avoid damage during the heating process.

Some of the other less frequently used well-known reducing sugar methods available are the CuBic[42] and the PJ[43] methods. As with the NS method, the CuBic method also measures the ability of the sugars to reduce copper in an alkaline medium. In this method, 2,2′-bicinchoninate is allowed to react with cuprous ions to form a purple complex, copper(I)–bicinchoninate, which absorbs light at 570 nm:[42,65]

$$\begin{gathered}\text{Sugar} + \text{Cupric ion}\left(Cu^{2+}\right) \rightarrow \text{Cuprous ion}\left(Cu^{+}\right) + \text{Sugar acid} \\ 2,2'\text{-Bicinchoninate} + \left(Cu^{+}\right) \rightarrow \text{Copper(I)} - \text{Bicinchoninate complex} \\ \text{(colourless)} \qquad\qquad \text{(purple)}\end{gathered} \tag{3}$$

The PJ method involves the reduction of ferricyanide ions in an alkaline medium to yield Prussian blue ferric ferrocyanide (Eqn (4)):[43]

$$\begin{aligned}&\text{Sugar} + \text{Ferricyanide ion}\left(\left[Fe(CN)_6\right]^{3-}\right) \rightarrow \text{Ferrocyanide ion}\left(\left[Fe(CN)_6\right]^{4-}\right) + \text{Sugar acid}\\ &\left[Fe(CN)_6\right]^{4-} + \text{Ferric ion}\left(Fe^{3+}\right) \rightarrow \text{Ferric ferrocyanide}\left(Fe_4\left[Fe(CN)_6\right]_3\right)\\ &\quad\text{(yellow)}\qquad\quad\text{(yellow)}\qquad\qquad\qquad\text{(Prussian blue)}\end{aligned} \tag{4}$$

Similar to the NS method, the CuBic method also shows a good stoichiometric relationship with the amount of reducing ends present in the medium[66] and is 100 times more sensitive than the DNSA method.[65] In contrast to the CuBic method, the PJ method also suffers from the same problem as the DNSA method, where the accuracy of the PJ assay is compromised by the size of the product formed. However, this problem was resolved in a study carried out by Hizukuri *et al*.,[67] where modifying the pH of the system was found to yield the same reducing effect as glucose for maltooligosaccharides up to a degree of polymerization (DP) of 40. Therefore, among the available reducing sugar methods, the NS, CuBic, and the modified PJ methods can be recommended for assessing α-amylase activity because they yield the same results for equimolar reduction of sugars regardless of their size.[56,67]

On the whole, one of the main disadvantages of the reducing sugar methods is that some of the principal chemicals used in these assays are toxic and harmful to health. For example, in the DNSA method, the DNSA and the reduced product ANS are toxic if swallowed and are corrosive and cause eye irritation. Furthermore, other chemicals, such as cyanide and molybdenum used in the NS, PJ, and CuBic methods are also toxic. Thus, careful measures should be taken during performing the assays to avoid any associated undesirable outcomes, and proper standards should be maintained during preparation, handling, and discharge of these chemicals. Another thing to be noted here is that most of these assays are not done on a microscale; as a result, a lot of chemical waste is produced during these assays. Thus, proper standards should be practiced during disposal of these chemical wastes.

Amyloclastic methods

As an alternative to the reducing sugar methods, amyloclastic methods have been used to determine α-amylase activity for a long time. The two most widely used amyloclastic methods are the starch–iodine[47,48] and the turbidity methods,[16] both of which assess the amount of starch consumed in a reaction by quantifying the unreacted starch in the assay medium. The iodometric method developed by Fuwa[47] is widely used to determine α-amylase activity[48,68] and to determine starch content in foods.[69,70] In this method, a combination of iodine (I_2)–potassium iodide (KI) solution is used to form the deep-blue-coloured starch–I_2 complex. The deep-blue colour results from the inclusion of the I_2 and tri-iodide (I_3^-) complex within the amylose helix structure.[71,72] When starch or amylose is acted upon by α-amylases, the size of the amylose chain decreases, consequently decreasing the size of the starch–I_2 complex and the blue intensity of the complex.[66] Thus, the basic principle of this assay is that, as the starch or amylose is hydrolysed by α-amylase into smaller carbohydrate units, the reduction in the intensity of blue colour is considered proportional to α-amylase activity. The colour of the starch–I_2 complex depends on the size of the amylose chain,[4,73,74] where, with an increase in chain length, the colour of the maltosaccharide–I_2 complex changes from brown (DP 21–24) to red (DP 25–29), red–violet (DP 30–38), blue–violet (DP 39–46), and finally blue (DP ≥47).[73] Amylose chains with ≤20 glucose units do not produce a colour with the I_3^-, whereas the amylopectin chain produces a red colour with the I_3^-.[71,72] However, the amylopectin chain does not contribute to the blue colour obtained from linear chains having ≥39 glucose units.[66]

The starch–I_2 method is quite frequently used to assess α-amylase activity due to its simplicity and long history of usage in the food industry. The common steps involved in the method are as follows. Step 1: addition of enzyme and starch into test tubes; step 2: addition of hydrochloric acid to stop the reaction; step 3: addition of distilled water; step 4: addition of the I_2:KI reagent; step 5: transfer of the reaction mixture into a cuvette or microtitre plate; and finally, step 6: measurement of absorbance. Most of the papers in the literature have utilized the cuvette method to do the test, where the reported test volume ranges from 20 to 200 mL.[47] In a study done by Xiao *et al*.,[48] the method was modified to a microtitre plate with a total volume of 200 μL. The starch–I_2 assays reported by different researchers are quite diverse with I_2 concentrations ranging from 0.25 mmol L^{-1} to 3 mol L^{-1} and with the wavelength used to measure colour development varying from 550 nm to 700 nm.[47,48] Some studies have used a single wavelength, whereas some other studies have adapted double or triple wavelengths to increase the accuracy of the assay.[72] However, this method requires a reliable pure amylose from different sources to construct a standard curve, which becomes problematic at times.[75]

As with the iodometric method, the turbidimetric method also measures the amount of substrate consumed in a reaction and is a rapid and simple technique. The turbidimetric method measures the amount of light absorbed by the remaining particles in the starch suspension after a certain period of time.[49] The decrease in turbidity is directly proportional to α-amylase activity. Thus, lower the turbidity of a solution higher is the enzyme activity. The steps involved in the turbidity method are as follows. Step 1: addition of enzyme and starch into test tubes/microtitre plate; step 2: transfer of the reaction mixture into a cuvette or microtitre plate; and finally, step 3: measurement of absorbance at 660 nm.[16] Turbidity measurements can be taken in a wide range of wavelengths (mostly between 620 and 700 nm) within the visible range as they do not

show a characteristic peak like the normal chromophores.[16] The method is adapted to measure α-amylase activity in blood,[49] food, and wastewater.[16] Amongst the α-amylase determination methods, turbidity is the simplest method with few assay steps and few chemical preparations. However, compared with the iodometric method, the turbidimetric method is less cited in the literature and is rarely applied for investigation of α-amylase activity. It should be noted that, despite its being used less, compared with the iodometric and other α-amylase assays discussed herein, the turbidimetric method is the most rapid, simple, easy, and most economical.

Compared with the reducing sugar methods, both the amyloclastic methods are quite simple and do not involve a heating step. One of the principal limitations of an I_2-based method is interference caused by I_2-reducing substances present in the samples.[68] For example, cysteine present in Luria broth was found to completely bleach the starch–I_2 blue colour at a concentration as low as 160 µmol L^{-1}.[68] Furthermore, as the starch–I_2 complex forms a deep blue colour, it poses difficulties in optimizing the control reading. Thus, measurement of a relatively small change in absorbance is quite challenging. Since the presence of excess starch can give negative data, to measure enzyme activity it is necessary to use accurate serial dilutions of starch. Moreover, as already discussed, the maltodextrins produced do not have equal complexing ability with I_2.[73] Since the colour of the complex changes based on the size of the dextrin chain, the colour intensity produced will depend on the maltodextrin composition of the sample. Therefore, the use of this technique to assess α-amylase activity might yield misleading results.

Unlike other α-amylase detection methods, for the turbidimetric method, the starch used should not completely dissolve but it should be able to form a suspension. Thus, it is essential to pipette out the same amount of starch into each well to get reproducible and reliable results. In a spectrophotometer, the remaining starch particles resulting from incomplete hydrolysis of starch reflect and scatter the incident light, thereby reducing the light intensity reaching the detector. This decrease is recorded as the absorbance value. Thus, if unequal amounts of starch are pipetted into the sample wells, this can result in artificially high absorbance values and compromise the accuracy of the test results. Sedimentation of the substrate and getting a homogeneous mixture into each well makes the method more difficult and less reliable. Although fast and relatively simple, the preparation of stable and reproducible starch solutions has limited the use of both these methods. Though both the amyloclastic methods are comparatively rapid and simple, these methods only give a qualitative indication of α-amylase activity and cannot be considered suitable for quantitative analysis. Furthermore, it should be noted that, in an enzyme reaction or any other chemical reaction, it is more convenient and reliable to measure the amount of product formed than measuring the amount of substrate consumed.

Enzymatic method

The enzyme glucose oxidase (GOD) is used to determine blood glucose level, total starch content in cereal products, and serum α-amylase activity because it is cheap, stable, and is highly specific for glucose. Since GOD is highly specific for glucose, the product of α-amylase (maltose and other dextrins; Eqn (5)) cannot be used directly with GOD to determine α-amylase activity and, therefore, is concomitantly hydrolysed by an amyloglucosidase to yield glucose (Eqn (6)):[76,77]

$$\text{Starch} \xrightarrow{\alpha\text{-Amylase}} \text{Maltose} + \text{Maltotriose} + \text{Dextrins} \quad (5)$$

$$\text{Maltose} + \text{Dextrins} \xrightarrow{\text{Amyloglucosidase}} \text{Glucose} \quad (6)$$

To quantify the amount of glucose produced, GOD is used along with peroxidase (POD) to determine the amount of glucose produced. The principle behind the method is that, at first, glucose is oxidized by GOD to yield gluconic acid and hydrogen peroxide (H_2O_2):

$$\text{Glucose} + O_2 \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2 \quad (7)$$

Next, the H_2O_2 generated is allowed to react with an electron acceptor, such as quinoneimine, in the presence of POD to form a pink-coloured product and its absorbance is measured at 500 nm:

$$2H_2O_2 + \text{Phenol} + \text{4-Aminoantipyrine} \xrightarrow{\text{POD}} \underset{\text{(Pink)}}{\text{Red quinone}} + 4H_2O \quad (8)$$

The intensity of pink colour formed is directly proportional to the concentration of glucose in the sample. The common steps involved in the method are as follows. Step 1: addition of α-amylase and starch into the microtitre plate; step 2: addition of a glucosidase; step 3: addition of GOD/POD reagent; step 4: measurement of absorbance at 500 nm.

The principal advantage of this method is that the use of an enzyme that is highly specific for glucose eliminates the interference from other materials and is able to yield highly accurate results. In a collaborative study carried out in 32 laboratories, the GOD/POD kit method was reported to show high accuracy and reproducibility in assessing the total starch content in cereals determined by quantifying the sugars produced through sequential hydrolysis by α-amylase and amyloglucosidase.[77] In addition to this, other advantages of the enzymatic method are that it does not involve any complex reagent preparation, no heating step is involved, and no long incubation period is required, thus making the method less complicated, less laborious, and less time consuming. The only reagent to be prepared in addition to the enzyme is the starch sample. Unlike the PJ, NS, and DNSA methods, the GOD/POD kit method does not involve any hazardous reagent preparation or handling. The method is also adaptable to microtitre plates since there is no heating step involved. To date, no reports on overoxidation of sugars has been reported, since whatever enzyme is used the final product measured is glucose through the hydrolysis by an amyloglucosidase. However, use of a glucosidase enzyme after digestion with α-amylase is a bit cumbersome and also makes the method a bit expensive due to the requirement of two expensive enzymes.

In a recent study, the GOD/POD kit has been successfully used to assess α-amylase activity directly.[33,46] That is, the requirement of a glucosidase to hydrolyse the product of α-amylase is omitted (Eqn (6)). The method is developed based on the interaction of GOD with maltose and other products of α-amylase action.[33,46] The basic principle behind the method is very simple, where the products of α-amylase interact with GOD in the presence of O_2 resulting in the production of H_2O_2, which is proportional to the amount of colour produced. Thus, the modified method only

involves three steps, as follows. Step 1: addition of α-amylase and starch into the microtitre plate; step 2: addition of GOD/POD reagent; step 3: measurement of absorbance at 505 nm.

The main concern regarding the method is the interaction of GOD with maltose and other products of α-amylase action. The specificity of GOD towards glucose is well documented.[78–80] According to reported literature, GOD does not show any significant interaction with non-glucose sugars.[81,82] In a study by Visvanathan *et al.*,[33] GOD was reported to show considerable interaction with maltose, where the sensitivity of GOD towards maltose was only 25-fold lower than glucose. The lowest detection limits for glucose and maltose were 0.01 mg mL^{-1} and 0.25 mg mL^{-1} respectively. Interestingly, compared to the DNSA method, the GOD/POD method shows higher sensitivity towards maltose, and it also shows a higher linearity for varying concentrations of acarbose than the DNSA method.[33] Furthermore, the GOD/POD method was also successfully used to measure α-amylase and α-amylase inhibitory activity of samples; and in a recently published paper, the interaction of products of α-amylase (maltose and other dextrins) with GOD has been proved without doubt through high-performance liquid chromatography (HPLC) studies.[46] However, despite the presence of the same number of reducing groups in glucose and maltose, there is a clear difference in the sensitivity of GOD towards glucose and maltose. Thus, the new method should be tested for its ability to produce identical reducing values for equimolar reduction of maltodextrins to ensure the accuracy of the method in assessing α-amylase activity. In addition, inhibition of the enzymes GOD and POD and the reduction of H_2O_2 by polyphenols is of key concern.[83]

Chromogenic methods

More recently, chromogenic methods have found widespread application in the determination of α-amylase activity, where a soluble dye or a chromogen is coupled to a substrate (maltooligosaccharides) to give a measurable optical signal when the chromogen is liberated as a result of α-amylase action. Most of these methods use modified starches or derivatives of starch, such as insoluble starch–dye complex (Phadebas, azo-starch)[84,85] or *p*-nitrophenyl (PNP)-glycosides-linked maltooligosaccharides (G3–G10)[86,87] as the substrates. The most important feature of these substrates is that they are specific for α-amylases and do not interact with other amylolytic enzymes.[88]

In the insoluble starch–dye method, a dye is covalently linked to starch or one of its components, such as amylose or amylopectin, to yield a coloured signal when hydrolysed by α-amylase. The dye-linked substrate remains water insoluble until it is attached to the dye; however, the free dye is water soluble. When these substrates are acted on by α-amylases, fragments containing dye are solubilized and the absorbance of the soluble fragments, which is considered a direct indication of α-amylase activity, is measured after removal of the unhydrolysed substrate via centrifugation.[15] The starch–dye method, particularly the Phadebas method is commercialized and is being widely used in the clinical, food, and forensic fields.[89] One of the main disadvantages of the starch–dye method is that the size of the starch hydrolysis product or the cleavage mechanism cannot be determined.[66] In addition, removal of the insoluble material through centrifugation is a bit cumbersome and has limited the widespread use of the Phadebas method.

In the defined substrate method, a chromogenic moiety, such as a PNP glycoside, is linked to maltooligosaccharides having three to ten glucose units.[87] Some of the commercially available α-amylase assay kits employ ethylidene–PNP–G7 and benzylidene–G7–α-PNP having seven glucose units as substrates. Here, the PNP moiety is covalently bound to the reducing end of the maltooligosaccharide through an α-1,4-hemiacetal linkage and the release of the yellow-coloured nitrophenol chromophore as a result of α-amylase action is measured either kinetically or in end-point assays. However, since α-amylase is only capable of cleaving the covalent bonds between adjacent glucose units, to release the chromogen from the coupled substrate an enzyme such as α^- or β-glucosidase is employed along with α-amylase. A special form of α-glucosidases is utilized in these kits, which are only capable of acting on low-DP maltooligosaccharides (DP 2–3) and has little action on the native substrate (DP 5–7).[77] When incubated with samples, α-amylase cleaves the long-chain PNP-linked maltooligosaccharide to shorter chain PNP-glycosides, which are then rapidly cleaved by α^- and/or β-glucosidases, releasing the yellow-coloured PNP chromophore, which is usually measured at a wavelength of 405 nm:[38,90]

$$\text{PNP-(Glucose)}_{4-10} \xrightarrow{\alpha\text{-Amylase}} \text{PNP-(Glucose)}_{(4-10)-n} + \text{(Glucose)}_n$$

$$\text{PNP-(Glucose)}_{1-3} \xrightarrow[\text{glucosidases}]{\alpha\text{- and }\beta\text{-}} \text{PNP} + \text{Glucose} \qquad (9)$$

The liberated form of the chromogen has the desired optical activity, which is correlated to the amount of α-amylase present in a test sample.

One of the main problems encountered in this method is the interference caused by other hydrolases (exoamylases, glucosidase, and glucoamylase), which are also capable of cleaving the PNP-linked substrate.[66] To overcome this problem, blocking agents are coupled at the non-reducing end of the PNP-linked substrate[91,92] so that in the absence of α-amylase no reaction will take place with no colour change (Fig. 2). For example, the 'Ceralpha' method developed by McCleary and Sheehan,[86] presently commercialized by Megazyme, employs a PNP-maltoheptoside blocked with a 4,6-*O*-benzylidene at the non-reducing end, thereby preventing the activity of other enzymes.[92] Any substance that is capable of preventing the activity of exoenzymes

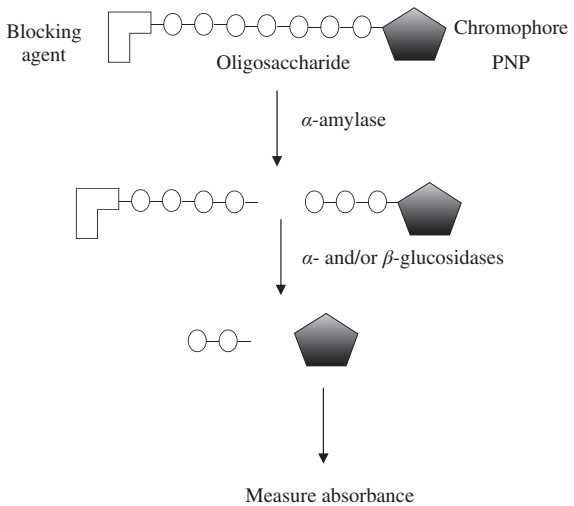

Figure 2. Activity of α-amylase and α^- and/or β-glucosidase on the blocking agent coupled PNP-linked substrate.

on the substrate can be used as blocking agents. The blocking agents act by disrupting the lock-and-key mechanism of the enzyme–substrate complex. In these kits, the blocking agents are normally coupled at the C2, C3, C4, or C6 of the terminal glucose, thereby making the terminal glucose unit incapable of fitting into the active site of the exoenzyme.

These methods are less time consuming, less laborious, easy, do not involve any toxic chemical handling, and, especially, are adapted to microtitre plates. Unlike the reducing sugar methods, these methods do not involve a heating step. Initially, the chromogenic methods were mainly developed to assess serum α-amylase activity. However, at present, these methods are widely used in different fields owing to their ease of use, accuracy, and high reproducibility. For example, the defined substrate method is widely used in the research field to measure α-amylase inhibitory activity. Many investigators at present use these methods to assess α-amylase activity due to the high accuracy of the results obtained through this method. Funke and Melzig[93] have adapted this method successfully to test several plant extracts for their inhibitory effects on porcine pancreatic α-amylase. The short detection wavelength used in this method is considered as an obstacle for assays determining α-amylase inhibitory activity, where the natural pigments, such as flavonoids and carotenoids, have the tendency to cause interference at that particular wavelength.[16]

The main concern of the defined substrate method is that the enzyme activity on short substrates (maltosaccharides) does not portray the actual situation, where the native starches may vary in structure and can be branched while the PNP-derivatized maltooligosaccharides are, indeed, too short to mimic the actual activity. The activity of the enzyme can vary depending on the composition and structure of the starch molecule.[94,95] For example, α-glucosidases are reported to show lower activity on long substrates than on short substrates.[66] Osman[88] reported the activity of α-amylase to differ based on the substrate, where the activity was only one-third that of β-amylase when measured with synthetic substrates but was higher with starch. The PNP-linked maltose derivatives were reported to have a larger K_m value than the native starch, indicating less affinity of the small substrates for α-amylase. This observation was explained by the presence of 7–11 subsites for sugar residues in the active site cleft of α-amylases, where the small substrates probably do not align properly in the active site to ensure maximal velocity.[94] Thus, many investigators have questioned the reliability of results obtained through these methods.[16,88]

RECOMMENDATIONS AND FUTURE PROSPECTS

α-Amylase catalyses the hydrolysis of internal α-1,4 glycosidic bonds in starch, resulting in the production of maltose, maltotriose, and α-limit dextrins. There are several methods in use utilizing different techniques to measure α-amylase activity, among which the spectroscopy-based methods are the most common. The reducing sugar and enzymatic, chromogenic, and amyloclastic methods are some of the most common spectroscopic methods in use. Each of these methods has its own advantages and disadvantages with respect to accuracy, reliability, convenience, and cost-effectiveness.

For example, though the DNSA method is comparatively convenient compared with the NS method, the method cannot be rated high in terms of accuracy and reliability. The non-equivalence reported between the amount of sugar reacted and ANS produced is a huge problem encountered in the DNSA method that compromises the accuracy and reliability. The same problem reported in the PJ method was resolved by modifying the pH. However, to date, no studies have reported a remedy to correct the problem encountered in the DNSA method. The DNSA method is assumed to rely on the reaction between DNSA and the carbonyl end of the reducing sugars. However, according to reported studies, the chemistry behind the DNSA method seems to be more complicated than just the production of ANS from DNSA, which requires further work. Despite the proven shortcomings associated with the DNSA method, it is still the most widely used method to measure α-amylase activity, mostly in the research field, which prompts reconsideration.

The remaining methods discussed in this paper also have their own pitfalls. In the I_2–starch method, the interference from I_2-reducing substances is an obstacle that needs to be resolved. This problem can be simply overcome by treating the sample with an oxidizing agent. But the disadvantage is that this pretreatment step will make the method more cumbersome and time consuming and, in some instances, might also affect the sample. In the chromogenic method, the main concern lies in the originality of the assay, where use of a short substrate (maltooligosaccharides composed of three to ten glucose units) instead of starch does not mimic the actual activity of α-amylase on starch. To surmount this shortcoming, such methods can be standardized against the rate of hydrolysis of a native substrate so that release of dyed fragments can be related to the rate of glycosidic bonds cleaved in a native substrate.

Until recently, most of these assays were done on a macroscale; as a result, a lot of chemicals and time were wasted while performing these assays. At present, the α-amylase methods have already been adapted to a microscale level, where several researchers have reported the use of these methods in microtitre plates, which saves time, chemical, and labour. As the next move, α-amylase detection methods are expected to step on to the dry-chemistry-based portable electrochemical or colorimetric sensors. Studies have reported the application of biosensors utilizing the DNSA,[96] I_2,[97] and GOD methods[17] with convincing outcomes, giving hope for future development. This technique is comparatively convenient and less time consuming, where the results can be obtained within a matter of seconds. This revolution is mainly expected in the food, textile, paper, and many other commercial industries where continuous process monitoring and control are required. The measurements can be considered as being mostly qualitative/semi-quantitative which is sufficient in most situations. However, the point-of-care measurements for α-amylase in the clinical set-up cannot be taken lightly, as a minute error in the reading might lead to devastating outcomes. The serum α-amylase level is strictly maintained within a narrow range, and semi-quantitative measurements cannot be considered reliable in clinical situations. Thus, for the time being, α-amylase activity measurements in the clinical field are expected to rely on laboratory-based spectroscopic methods for confirmed diagnosis. Some studies have reported the quantitative applicability of biosensors for serum amylase detection with limited commercial success.[96,98,99] Furthermore, the research field is also expected to almost rely on the spectroscopic methods for quantitative determination of α-amylase activity. However, future research will continue to work towards developing sensors with high accuracy and sensitivity, and very soon the development of sensors with the required desired characteristics can be expected.

Other than this, HPLC coupled to a refractive index (RI) detector or high-performance anion exchange (HPAE) coupled with pulsed amperometric detection (PAD) can be used to quantitatively detect α-amylase activity based on the amount of individual sugars produced. Visvanathan *et al.*[46] studied the products of α-amylase through the HPLC-RI method, whereas the application of HPLC for detecting α-amylase inhibitory activity has been discussed by Takács *et al.*[100] Recently, Pyner *et al.*[101] discussed the application of the HPAE-PAD method to assess sucrase-isomaltase activity. The method was optimized to detect the amount of glucose produced after sucrase-isomaltase activity. The same principle can be applied to assess α-amylase activity using the standards maltose and other dextrin units. However, the huge initial instrumental and day-to-day operational costs along with requirement of trained personnel have limited the widespread application of this technique.

Overall, it can be said that, despite the presence of numerous methods, there is no simple universal method by which α-amylase activity can be assessed easily, accurately, and quantitatively. As a result, many improvements in the α-amylase assay techniques have been made in recent years and are yet to be made in the years to come to get more accurate and reliable results. Thus, for the time being, we suggest selecting the appropriate method for the intended application while considering the benefits and shortcomings associated with each method.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this article. All of the authors reviewed the paper and approved the final version submitted for publication.

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