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 easiness 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. Furthermore, 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 catalyze 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] It acts at random locations of the starch molecule yielding oligosaccharides with varying chain length, which constitute a mixture of maltose, maltotriose, and branched oligosaccharides of 6–8 glucose units that contain both α-1,4 and α-1,6 linkages (Figure 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 food stuffs 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 is 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 is used 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 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.

There are several methods reported in literature to measure α-amylase activity. α-Amylase is probably the enzyme with the most published analytical procedures to assay its catalytic activity. 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 easiness. In 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 easiness and cost effectiveness. Thus, the present 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 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.[22] The quality of starch-containing foods is significantly influenced by the action of naturally present α-amylase in foods, which can hydrolyze starch and eventually result in viscosity loss. Starch is mainly used as a thickening agent in many food preparations such as sauce, soups and canned stews and curries.[8] Presence of even a small amount of α-amylase can hydrolyze 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] Presence of high levels of α-amylase in flours reduced bread volume, breadcrumb strength and increased the levels of starch degradation products resulting in a sticky crumb.[10,24] On the other hand, very low levels of α-amylase is also associated with

reduced loaf volume, poor quality crumb and reduced shelf life of bread.[24] Thus, α-amylase activity monitoring is indispensable to maintain 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 soils from cloths.[2,11] α-Amylase is used in wastewater treatment plants to hydrolyze 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 remedy 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 hydrolyzed 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 are further hydrolyzed 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 the 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.[36–38] α-amylase in human body is present in two isoforms; P-type and S-type.[7,36] 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.[36] The major production site of S-type α-amylase is the salivary glands, where it initiates the hydrolysis of starches while food is in the mouth and esophagus and its action is terminated by acid in the stomach. About 20% of α-amylase in the plasma is excreted by the kidneys[38] and the major catalytic site of the remainder is the liver.[36] The normal range for serum α-amylase activity is approximately 36-128 Unit/L.[37] 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, macroamylasemia, mumps, renal failure and other malignant conditions such as breast, colon, lung, and ovarian cancers.[12,37] 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.[39–41]

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: (a) decrease of viscosity of a starch solution, (b) decrease of turbidity of a starch suspension, (c) decrease of intensity of a starch-iodine reaction and (d) 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

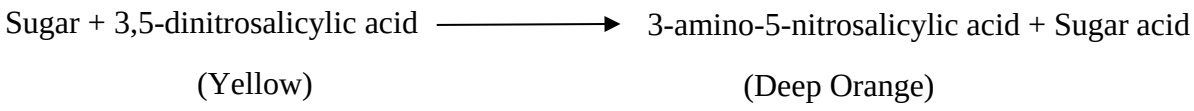
A number of reducing methods are used to detect α-amylase activity including the 3,5-dinitrosalicylic acid (DNSA),[13] Nelson-Somogyi (NS),[42] copper-bicinchoninate (CuBic)[43] and the Park and Johnson (PJ)[44] method. All these methods are based on measuring the oxidizing ability of reducing sugars. 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 sugar is buried in the glycosyl bond making it incapable of acting as a reducing agent anymore.[45]

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.[13,46] The method was later optimized by Noelting & Bernfeld[47] to measure reducing sugars released by the action of α-amylase and has also been widely used to measure other carbohydrase activities.[48–50] 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 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 3,5-dinitrosalicyic acid with the carbonyl end of the reducing sugars to yield the deep orange

coloured 3-amino-5-nitrosalicylic acid (ANS) (Eq.1), which absorbs light strongly at 540 nm. In other words, while the 3,5-dinitrosalicylic acid is reduced to 3-amino-5-nitrosalicylic acid, the functional groups (aldehyde/ketone) of the sugars are simultaneously oxidized to its respective carboxylic acids. Thus, theoretically the amount of 3-amino-5-nitrosalicylic acid 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 3-amino-5-nitrosalicylic acid produced.[45,51–53] This problem was first reported in a study done by Robyt & Whelan,[52] 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 over-oxidation 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 & Whitney,[45] 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.[45,51] Thus, the use of the DNSA method for the determination of α-amylase gives only the 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.[54] Furthermore, the presence of some amino acids[55] as well as polyphenols[56] have been reported to interfere with the assay. The interference from polyphenols was corrected by passing the reaction mixture through a solid phase extraction unit.[56]



Eq: 01

Some of the other main disadvantages of the DNSA method are, 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.[57] 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 micro level facilitates rapid screening of a large number of samples. Two papers have discussed the adaptation of the

DNSA method to microtitre plates.[57,58] 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 Nelson-Somogyi method (NS),[42,59] 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 (Eq.2).[55] The steps involved in the NS method are, 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.

$$\text{Sugar} + \text{Cupric ion } (Cu^{2+}) \longrightarrow \text{Cuprous ion } (Cu^{+}) + \text{Sugar acid}$$

$$\underset{\text{(Colourless)}}{\text{Arsenomolybdate complex} + Cu^{+}} \longrightarrow \underset{\text{(Blue)}}{\text{Molybdenum blue} + Cu^{2+}}$$

Eq: 02

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.[53] The over-oxidation problem encountered in the DNSA method was not

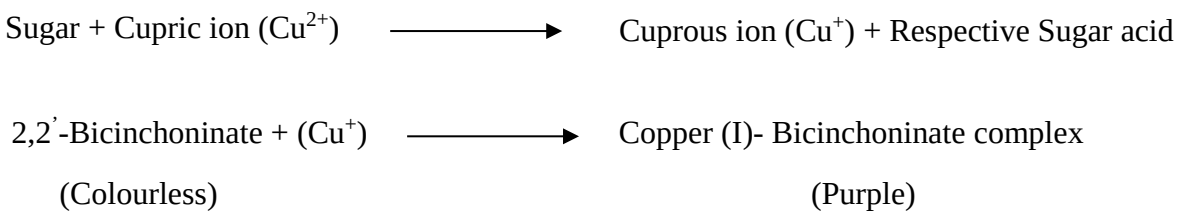
observed in the NS method in a study done by Robyt & Whelan,[52] where the reducing value of maltose, malto-oligosaccharides, and maltodextrins showed an apparent stoichiometric relationship with the available hemiacetal reducing groups. In addition, the NS method is reported to be 10-times more sensitive than the DNSA method.[50,52] However, care should be taken to prevent re-oxidation of Cu(I) to Cu(II) during the heating and cooling period before arsenomolybdate reagent addition, as the back-oxidation have a high possibility of re-oxidation.[60]

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 discrepancy 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 3,5-dinitrosalicylic acid reagent for 5 min, followed by cooling and absorbance reading.[13,46] 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 Nelson-Somogyi 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.[53,61]

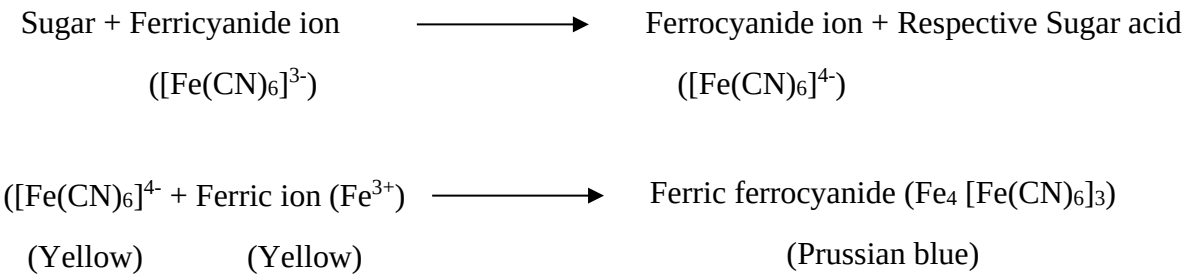
Both the NS and the DNSA method require a large number of reaction tubes and transfer of the final solution to cuvettes/microtitre plate for absorbance reading. In addition, handling a large number of tubes during the heating process (in a water bath) limits the analytical capacity (number of samples that can be assayed at a time) and efficiency of the methods. To overcome this problem, recently Shao & Lin[54] developed a more convenient microplate based NS method for the determination of reducing sugars which has significantly reduced the assay time

(approximately within one hour 25 samples can be assayed), 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 damages during the heating process.

Some of the other less frequently used well known reducing sugar methods available are the copper-bicinchoninate (CuBic)[43] and the Park and Johnson (PJ)[44] methods. Same as in the NS method, the CuBic method also measures the ability of the sugars to reduce copper in an alkali 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 (Eq. 03).[43,62] The PJ method involves the reduction of ferricyanide ions in an alkaline medium to yield Prussian blue ferric ferrocyanide (Eq. 04).[44] Similar to the NS method, the CuBic method also shows a good stoichiometric relationship with the amount of reducing ends present in the medium[63] and is 100-times more sensitive than the DNSA method.[62] 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.[64] where modifying the pH of the system was found to yield the same reducing effect as glucose for malto-oligosaccharides up to a DP (Degree of polymerization) 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 same results for equimolar reduction of sugars regardless of their size.[52,64]



Eq: 03



Eq: 04

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 for health. For example, in the DNSA method, the dinitrosalicylic acid and the reduced product, 3-amino-5-nitro-salicylic acid are toxic if swallowed and are corrosive and causes eye irritation. Furthermore, other chemicals such as cynanide and molybdenum used in the NS, PJ and CuBic method 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 micro scale and 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 are been used to determine α-amylase activity for a long time. The two most widely used amyloclastic methods are the starch-iodine[65,66] and the turbidity method[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[65] is widely used to determine α-amylase activity[66,67] and to determine starch content in foods.[68,69] In this method, a combination of I_2-KI solution is used to form the deep blue coloured starch-iodine complex. The deep blue colour results from the inclusion of the iodine and tri-iodide (I_2 and I_3^-) complex within the amylose helix structure.[70,71] When starch or amylose is acted upon by α-amylases, the size of the amylose chain decreases, consequently decreasing the size of the starch-iodine complex and the blue intensity of the complex.[63] Thus, the basic principle of this assay is that as 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-iodine complex depends on the size of the amylose chain[4,72,73] where with an increase in chain-length, the colour of the maltosaccharide-iodine 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).[72] Amylose chains with 20 or less glucose units do not produce a colour with the tri-iodide ion, whereas, the amylopectin chain produces a red colour with the tri-iodide ion.[70,71] However, the amylopectin chain does not contribute to the blue colour obtained from linear chains having 39 glucose units or more.[63]

The starch-iodine 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, step 1: addition of enzyme and starch into test tubes; step 2: addition of HCl 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 literature have utilized the cuvette method to do the test where the reported test volume range from 20 to 200 mL.[65] In a study done by Xiao et al.,[66] the method was modified to a mictotire plate with a total volume of 200 µL. The starch-iodine assays reported by different researchers are quite diverse with iodine concentrations ranging from 0.25 mM to 3 M and with the wavelength used to measure colour development varying from 550 nm to 700 nm.[65,66] Some studies have used one single wavelength while some other studies have adapted double or triple wavelengths to increase the accuracy of the assay.[71] However, this method requires a reliable pure amylose from different sources to construct a standard curve, which becomes problematic at times.[74]

Same as the iodometric method, the turbidimetric method also measures the amount of substrate consumed in a reaction and is a rapid and simple technique. Turbidimetric method measures the amount of absorbed light by the remaining particles in the starch suspension after a certain period of time.[75] 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, 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 the wavelengths of 620-700 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[75], food and wastewater treatment.[16] Amongst the α-amylase determination methods, turbidity is the simplest method with few assay steps and few chemical preparations. However, compared to the iodometric method, the turbidimetric method is less cited in literature and is rarely applied for investigation of α-amylase activity. It should be noted that despite its less usage, compared to the iodometric and other α-amylase assays discussed above, turbidimetric method is the most rapid, simple, easy and the most economical method.

Compared to 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 iodine-based method is interference caused by iodine-reducing substances present in the samples.[67] For example, cysteine present in Luria broth was found to completely bleach the starch-iodine blue colour at a concentration as low as 160 μm.[67] Furthermore, as the starch-iodine 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 presence of excess starch can give negative data, to measure enzyme activity, accurate usage of serial dilutions of starch is necessary. Moreover, as discussed above, the maltodextrins produced don't have equal complexing ability with iodine.[72] Since the colour of the complex changes based on the size of the dexrtrin 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 lead to misleading results.

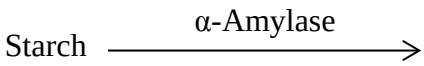
Unlike other α-amylase detection methods, for the turbidimetric method, the starch used should not completely dissolve but it should be able to form a suspension. Thus, it is essential to pipette

out same amount of starch in to 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 amount of starch is pipetted into the sample wells, this can result in artificially high absorbance values, compromising the accuracy of the test results. 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 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.

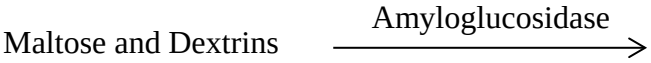
3.3 Enzymatic method

The enzyme glucose oxidase (GOD) is used to determine blood glucose level, total starch content in cereal products and to determine 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) (Eq. 05) cannot be used directly with GOD to determine α-amylase activity, and is therefore concomitantly hydrolyzed by an amyloglucosidase to yield glucose (Eq. 06).[76,77] To quantify the amount of glucose produced, glucose oxidase is used

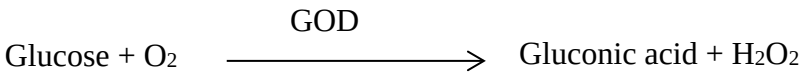
along with peroxidase (POD) to determine the amount of glucose produced. The principle behind the method is that at first, glucose is oxidized by GOD to yield gluconic acid and hydrogen peroxide (Eq. 07). Next, the generated hydrogen peroxide is allowed to react with an electron acceptor such as quinoneimine in the presence of peroxidase to form a pink coloured product and its absorbance is measured at 500 nm (Eq. 08). 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, 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.



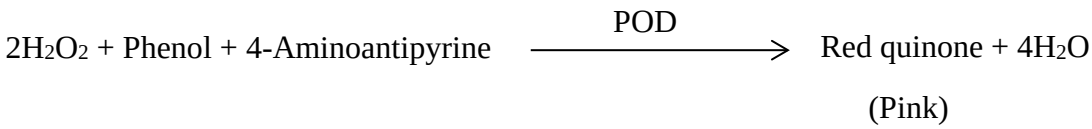
Eq: 05



Eq: 06



Eq: 07



Eq: 08

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, no long incubation period thus making the method less complicated, less labourious 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 method, the GOD/POD kit method does not involve any hazardous reagent preparation or handling. The method is also adaptable to microtitre plate since there is no heating step involved. To date no reports on over-oxidation of sugars is reported since, whatever the 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.[33,78] That is, the requirement of a glucosidase to hydrolyze the product of α-amylase is omitted (Eq. 06). The method is developed based on the interaction of glucose oxidase with maltose and other products of α-amylase action.[33,78] 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 hydrogen peroxide, which is proportional to the amount of colour

produced. Thus, the modified method only involves 03 steps, 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.[79–81] According to reported literature, GOD does not show any significant interaction with non-glucose sugars.[82,83] In a work done 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 and 0.25 mg/mL, respectively. Interestingly, compared to the DNSA method, the GOD/POD method showed higher sensitivity towards maltose and also it showed 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 doubts through HPLC studies.[78] However, despite the presence of 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, glucose oxidase and peroxidase and the reduction of hydrogen peroxide by polyphenols is of key concern.[84]

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 (malto-oligosaccharides) 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)[85,86] or p-nitrophenyl glycosides linked malto-oligosaccharides (G3-G10)[87,88] as the substrates. The most important feature of these substrates is that these substrates are specific for α-amylases and does not interact with other amylolytic enzymes.[89]

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 and when these substrates are acted on by α-amylases, fragments containing dye are solubilized and the absorbance of the soluble fragments, which is considered as 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 field.[90] One of the main disadvantage of the starch-dye method is that the size of the starch hydrolysis product or the cleavage mechanism cannot be determined.[63] 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 p-nitrophenyl (PNP) glycoside is linked to malto-oligosaccharides having glucose units of 3 to 10.[88] Some of the commercially available α-amylase assay kits employ ethylidene-pNP-G7 and benzylidene-G7-α-PNP having 7 glucose units as substrates. Here the p-nitrophenyl moiety is covalently bound to the reducing end of the malto-oligosaccharide 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-glucosidase 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 malto-oligosaccharides (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 malto-oligosaccharide 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 the wavelength of 405 nm (Eq.9).[39,91] 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.

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

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

Eq: 09

One of the main problems encountered in this method is the interference caused by other hydrolases (exo-amylases, glucosidase and glucoamylase), which are also capable of cleaving the PNP linked substrate.[63] To overcome this problem, blocking agents are coupled at the non-reducing end of the PNP linked substrate[92,93] so that in the absence of α-amylase no reaction will take place with no colour change (Figure 2). For example, the 'Ceralpha' method developed by McCleary & Sheehan,[87] presently commercialized by Megazyme employs a p-NP-maltoheptoside blocked with a 4,6-O-benzylidene at the non-reducing end, thereby, preventing the activity of other enzymes.[93] Any substance that is capable of preventing the activity of exo-enzymes 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 in to the active site of the exo-enzyme.

These methods are less time consuming, less labourious, 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 its easiness, 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 high accuracy of results obtained through this method. Funke & Melzig[94] have adapted this method successfully to test several plant extracts for their inhibitory

effects on porcine pancreatic α-amylase. The short detection wavelength occupied in this method is considered as an obstacle for assays used to determine α-amylase inhibitory activity, where the natural pigments such as flavonoids and carotenoids have the tendency to cause interference in 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 p-NP-derivatised malto-oligosaccharide 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.[95,96] For example, α-glucosidases are reported to show lower activity on long substrate than on short substrates.[63] Osman[89] reported the activity of α-amylase to differ based on the substrate, where the activity was only one-third than 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 Km 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 sub sites 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.[95] Thus many investigators have questioned the reliability of results obtained through these methods.[16,89]

Recommendations and future prospects

α-Amylase catalyzes the hydrolysis of internal α-l,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 spectroscopic based methods are the most common. The reducing sugar, enzymatic, chromogenic and the 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 than the NS method, in terms of accuracy and reliability the method cannot be rated high. Non-equivalence reported between the amount of sugar reacted and 3-amino-5-nitrosalicylic acid 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, still it's the most widely used method to measure α-amylase activity, mostly in the research field, which prompts reconsideration.

The remaining methods discussed in the present paper also have their own pitfalls. In the iodine-starch method, the interference from iodine 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 on the originality of the assay where use of a short substrate (malto-oligosaccharides composed of 3 to 10 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 hydro,lysis 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 recent time, most of these assays were done on a macro scale and as a result a lot of chemical and time was wasted while performing these assays. At present, the α-amylase methods have already been adapted to a microscale level where several authors have reported the use of these methods in microtiter 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 colourimetric sensors. Studies have reported the application of biosensors utilizing the DNSA[97], iodine[98] and GOD method[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 is required. The measurements are objective and qualitative/semi quantitative measurements can be considered 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, but with limited commercial success [97,99,100]. 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, high-performance liquid chromatography (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.[78] studied the products of α-amylase through HPLC-RI method whereas the application of HPLC for detecting α-amylase inhibitory activity have been discussed by Takács.[101] Recently Pyner et al.[102] have 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. 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 cost along with requirement of trained personnels 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 on the years to come to get more accurate and

reliable results. Thus, for the time being, we suggest to select 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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Table 1. Brief overview on α-amylase detection methods

	Reducing sugar methods	**Enzymatic method (GOD/POD)**	**Chromogenic method**	**Iodine-starch method**	**Turbidimetric method**
Assay principle	Based on the oxidizing ability of sugars	Based on the interaction of glucose/products of α-amylase with glucose oxidase	Interaction of the substrate with α-amylase and release of the chromophore upon hydrolysis by a α-glucosidase	Based on the interaction of iodine and tri-iodide (I_2 and I^-_3) ion with the amylose helix structure	Based on the reduction of turbidity of a starch solution due to breakdown of starch
Examples	DNSA, Nelson-Somogyi, PJ, CuBic	GOD/POD method	Phadebas, Ceralpha	Iodine-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, AZCL 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	5-20 min	5-15 min	10-15 min	-	-
Dilution	√	-	-	Sometimes required	-
Total assay steps	6-8	3-4	4	6	2/3
Minimum detection limit	DNSA-2.92 mM* NS- 0.03 mM** CuBic-5 µM* PJ- 5 µM**	0.73 mM*	1.43 U/L***	N/A	1 U/L***
Absorbance reading	540-620 nm	500-505 nm	405 nm	550-700 nm	600-660nm
References	13,42-44,51,54	33,78	40	65,66	16,75

Minimum detection limit in terms of *Maltose, **Glucose, and ***amylase enzyme activity

N/A-Not available

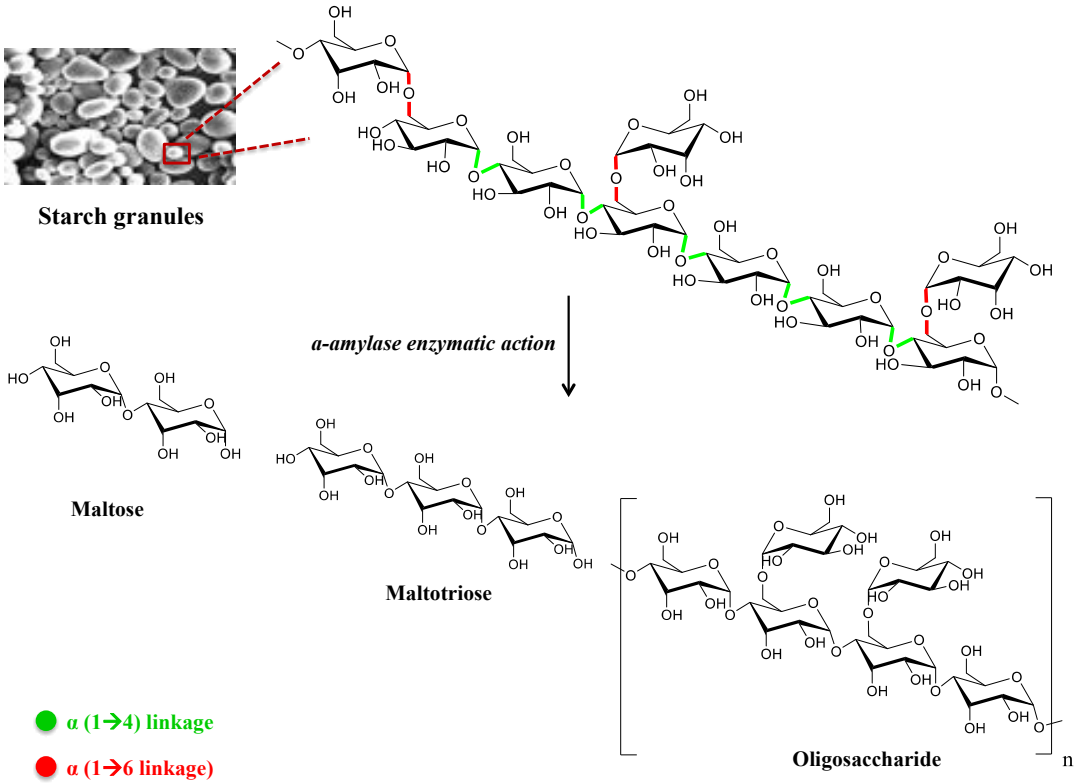

Figure 1. Graphical representation of α-amylase action on starch

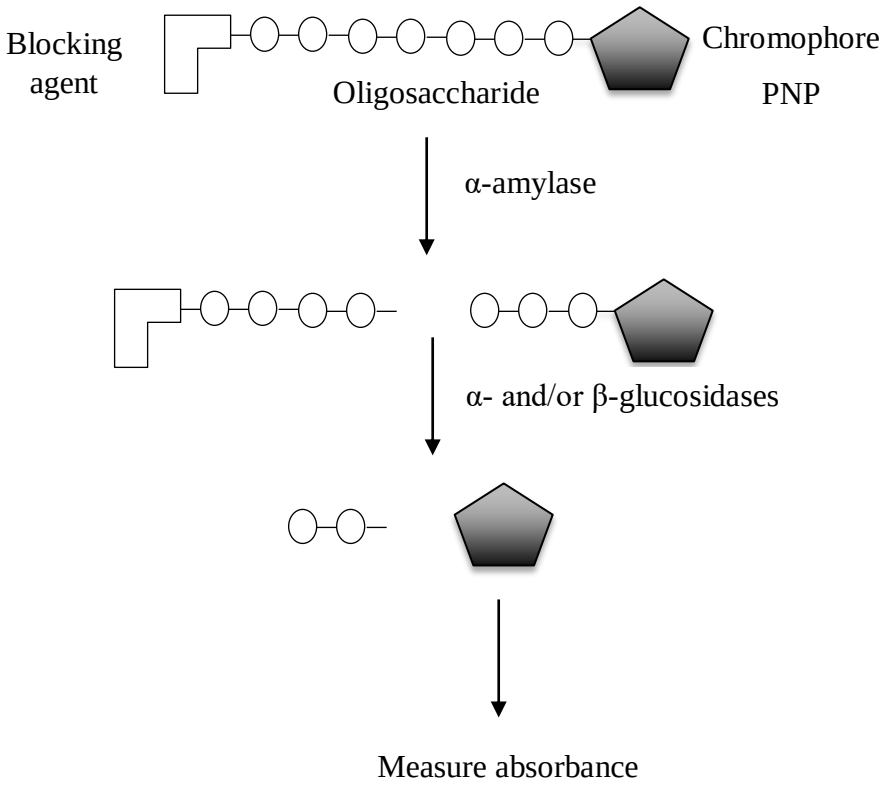

Figure 2. Activity of α-amylase and α- and/or β-glucosidase on the blocking agent coupled PNP linked substrate