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Modified bicinchoninic acid assay for accurate determination of variable length reducing sugars in carbohydrates

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Abstract

The accurate determination of reducing ends of malto-oligosaccharides is essential for calculating the enzyme activities of starch debranching enzymes. The suitability of the 3,5-Dinitrosalicylic acid (DNS) method, the Dygert method, and the Bicinchoninic acid (BCA) method for accurate determination of reducing ends from malto-oligosaccharides of different chain lengths is compared. The results showed that BCA assay was much more accurate than the other assays. The results for the BCA assay showed that different malto-oligosaccharides gave observed (measured) values that were significantly similar to the expected (predetermined) values. In contrast, the DNS and Dygert assays underestimated the amount of reducing sugar present for glucose. Furthermore, both DNS and Dygert methods showed increasing degree of overestimation of the amount of reducing sugar present with the increasing length of the malto-oligosaccharide sugar chains. The BCA assay can suitably quantify reducing sugars even in mixtures of oligosaccharides with different chain lengths. Thus, enzyme activities can be measured without bias towards higher values for enzymes that preferentially cleave the longer chain lengths.

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Introduction

Linear malto-oligosaccharides with various chain length are products of hydrolysis of starch by several starch debranching enzymes such as isoamylase and pullulanase. Starch debranching enzymes play an important role in the synthesis of amylopectin (Zeeman *et al*., 2010). They hydrolyse the α-1,6-branches in glucan (Hussain *et al*., 2003), producing linear malto-oligosaccharides (González, 1994). The accurate determination of reducing ends of these malto-oligosaccharides is essential for calculating enzyme activity of debranching enzymes. Among the current methods used to quantify reducing ends of sugars are the 3,5-Dinitrosalicylic acid (DNS) method, the Dygert method, and the Bicinchoninic acid (BCA) method. However, enzyme activities may be over or under represented depending on the chain length of the malto-oligosaccharides measured. It has been reported that DNS reducing sugar assay results may have been misinterpreted in literature as it shows differential behaviour for sugars of different chain lengths (Saqib and Whitney, 2011; McIntyre *et al.*, 2013).

The DNS method, which is among the most common methods to quantify reducing sugars (Miller, 1959; Bailey *et al.*, 1992; Gonçalves *et al.*, 2010; Silveira *et al*., 2014), involves the reduction of a nitro group (NO_2) in 3,5 dinitrosalicylic acid to an amine group (NH_2), in alkaline condition, producing 3-amino-5-nitrosalicylic acid. The reaction requires an aldehyde group (CHO) present in the reducing agent (i.e. DTT and reducing sugars), which is oxidised to a carboxyl group (COOH). This reduction produces a coloured solution, which is stabilised by tartrate ions (Miller, 1959). The colour intensity can be measured at 540 nm.

The Dygert method is another method used to measure reducing sugar (Dygert *et al*., 1965; Han *et al.,* 2012; Kotake *et al*., 2013). The assay is based on the reduction of ion Cu^{2+} to Cu^{+} by reducing sugars. The subsequent reaction of ion Cu^{+} and neocuproine in alkaline condition forms a coloured complex that can be read at 405 nm (Dygert *et al.,* 1965).

The BCA method is mainly used in protein quantification (Smith *et al*., 1985; Horn and Eijsink, 2004; Huang *et al*., 2010) but has also been adapted to measure reducing sugars (Hussain *et al.*, 2003; Utsumi *et al*., 2009; Nadour *et al.*, 2015). The reaction with reducing agents results in the reduction of Cu^{2+} to Cu^{+}, followed by the chelation of one Cu^{+} with two BCA molecules, forming an intense

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purple colour with an absorbance maximum at 562 nm (Walker, 2002; Huang *et al.*, 2010). The BCA assay is highly sensitive for reducing ends and it is also generally more tolerant to interference from other compounds (Walker, 2002). Most importantly, optimized conditions of the BCA assay have been shown to give relatively uniform values regardless of the degree of polymerisation of the oligosaccharide (Doner and Irwin, 1992).

In this paper, a comparison of the accuracy of DNS, Dygert and BCA reducing sugar assays for quantification of reducing ends from malto-oligosaccharides with different chain lengths is described.

Materials and methods

Malto-oligosaccharides

The malto-oligosaccharides used for comparisons between different assay methods were of high purity (> 95%) glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6) and maltoheptaose (G7), from Megazyme. All malto-oligosaccharides used in different assay methods were prepared in the predetermined (expected) concentrations based on their molecular relative mass prior to the assays. This known, predetermined concentrations would be compared against the observed (measured) concentrations based on the absorbance value that was plotted using maltose standards for each different assay. For DNS and BCA assays, each assay method was conducted at two different known predetermined malto-oligosaccharide concentrations: 0.4 and 1.0 µmol/assay while for Dygert assay, predetermined concentrations were 0.8 and 2.0 µmol/assay.

DNS reducing sugar assay

This assay is based on the protocol described by Bernfeld (1951). DNS reagent was prepared by mixing 1.6 g NaOH and 1.0 g dinitrosalicylic acid (Sigma) in 70 mL dH_2O and the mixture was heated in boiling water to dissolve. Once dissolved, 3.0 g Na_2K tartrate (Sigma) was added to the solution and swirled until dissolved followed by further addition of dH_2O to make up to 100 mL. The reagent was stored dark at room temperature. For the assay, 100 µL of each malto-oligosaccharide solution at known concentration described above was added to 100 µL DNS reagent. The mixture was then boiled for exactly 5 mins, vortexed for few seconds and centrifuged at 14,000 X *g* for 10 mins. Two aliquots of 90 µL from each duplicate assay were used to measure the absorbance at 540 nm. Measured concentration was then determined by plotting the absorbance value against the maltose standard prepared using the same assay method.

BCA reducing sugar assay

This assay is based on the methods of Fox and Robyt (1991) and Meeuwsen *et al.* (2000) with a few modifications such as the assay volume, incubation time and wavelength. The BCA reagent consisted of stock solution A (0.5 M Na_2CO_3, 288mM $NaHCO_3$, 5mM sodium bicinconinic acid [Sigma]) and stock solution B (12 mM L-serine, 5mM $CuSO_4.5H_2O$). Stock solutions of A and B were mixed at ratio of 1:1 before being used. For the assay, 500 µL of each malto-oligosaccharide solution at known concentration described above was added to 500 µL of BCA reagent. The mixture was incubated at 80°C for 1hr and cooled to room temperature. Two aliquots of 200 µL from each duplicate assay were used to measure the absorbance at 562 nm. Measured concentration was then determined by plotting the absorbance value against the maltose standard prepared using the same assay method.

Dygert reducing sugar assay

This assay is based on the protocol by Dygert *et al.* (1965) with few modifications. Dygert reagent consisted of two assay solutions. Solution A consisted of 4% (w/v) Na_2CO_3, 0.16% (w/v) glycine and 0.045% (w/v) $CuSO_4.5H_2O$. Solution B consisted of 0.12% (w/v) neocuproine-HCl. For the assay, 75 µL of each malto-oligosaccharide solution was mixed with 25 µL of assay buffer [400 mM MOPS pH 7.0, 20% v/v ethanediol, 40 mM EDTA, 8 mM DTT, 200 µg/mL Acarbose (Bayer)]. The mixture was incubated at 30°C for 1hr. The reaction was stopped by adding 100 µL solution A and 100 µL solution B. The mixture was vortexed, boiled for 12 mins and cooled. This was followed by addition of 100 µL of ethanol to the assay, vortexed for few seconds and centrifuged at 14,000 X *g* for 10 mins. Two aliquots of 100 µL from each duplicate assay were used to measure the absorbance at 405 nm. Measured concentration was then determined by plotting the absorbance value against the maltose standard prepared using the same assay method.

Results and discussion

The malto-oligosaccharides tested represented simple sugar molecules, from a sugar molecule with one glucose ring (G1) up to a sugar molecule with seven glucose rings (G7). Although these different types of malto-oligosaccharides do not represent all

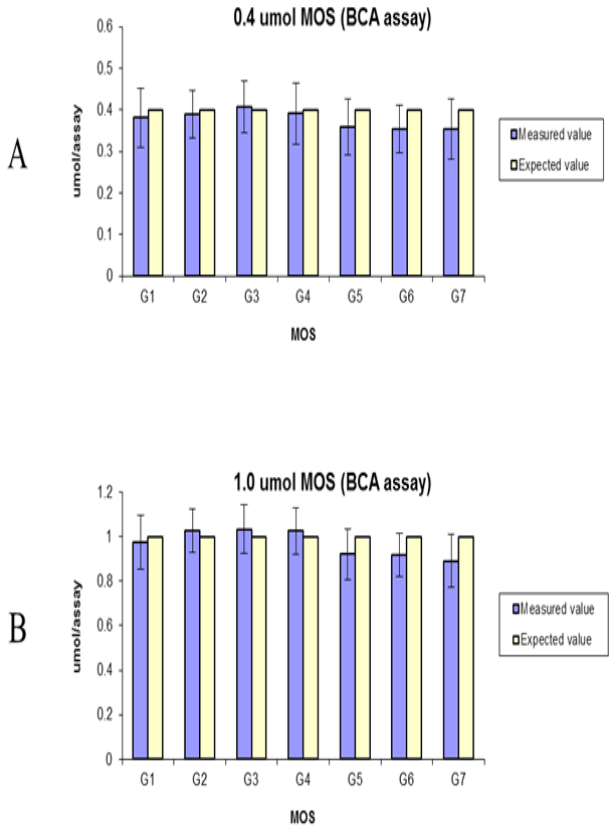

Figure 1. Measured and expected value of different malto-oligosaccharides (MOS) concentration using BCA assay. A; Measured values compared to the expected value at 0.4 µmol MOS/assay. B; Measured values compared to the expected value at 1.0 µmol MOS/assay. G1 to G7 indicate MOS with one glucose ring (G1) up to MOS with seven glucose rings (G7). Error bars represent ± standard deviations from two duplicates of assay.

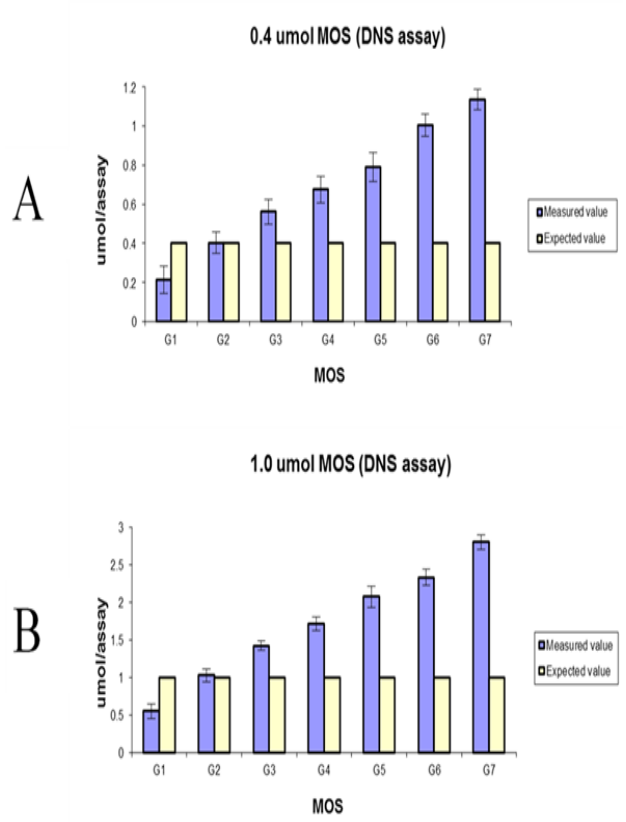

Figure 2. Measured and expected value of different malto-oligosaccharides (MOS) concentration using DNS assay. A; Measured values compared to the expected value at 0.4 µmol MOS/assay. B; Measured values compared to the expected value at 1.0 µmol MOS/assay. G1 to G7 indicate MOS with one glucose ring (G1) up to MOS with seven glucose rings (G7). Error bars represent ± standard deviations from two duplicates of assay.

the degrees of polymerisation of sugar chains that may be the products of enzyme activities (such as isoamylase and other types of debranching enzymes) on starch substrates which can be of higher sugar chains for the amylopectin substrates, these malto-oligosaccharides can basically provide an indication whether different chain lengths of sugar will give a same absorbance unit if they are present at the same concentration.

The results for the BCA reducing sugar assay showed that different types of malto-oligosaccharides gave measured values that were significantly similar to the expected (predetermined) values at both the 0.4 µmol and 1.0 µmol concentrations (Figure 1). At the expected value of 0.4 µmol, the lowest measured value was 0.353 µmol from G6 while the highest measured value was 0.407 µmol from G3. The lowest measured value for the expected value of 1.0 µmol was 0.891µmol that was given by G7 and the highest measured value was 1.032 µmol by G3. For all types of malto-oligosaccharides, the error bar(s) of the measured values overlapped with the expected values, thus there were no significant differences between the measured and expected values at both concentrations. This indicates that the BCA assay provides fairly accurate measurement for different types of malto-oligosaccharides.

The DNS assay showed that only maltose returned the same figures for the measured and expected values at both 0.4 µmol and 1.0 µmol concentrations. Glucose gave a lower value for the measured value while other malto-oligosaccharides, gave an increasing value from G3 onwards at both 0.4 and 1.0 µmol concentrations (Figure 2). This meant that the higher degree of polymerisation gave a higher concentration value although the expected value was much lower. A similar observation has been reported previously (McIntyre *et al.*, 2013) where increasing absorbance values were obtained with increased oligosaccharide chain length because oxidation did not stop with the reducing-end glucose but instead continued down the chain, resulting in over-oxidation of the oligosaccharide. Therefore, the DNS assay is more suitable for a qualitative

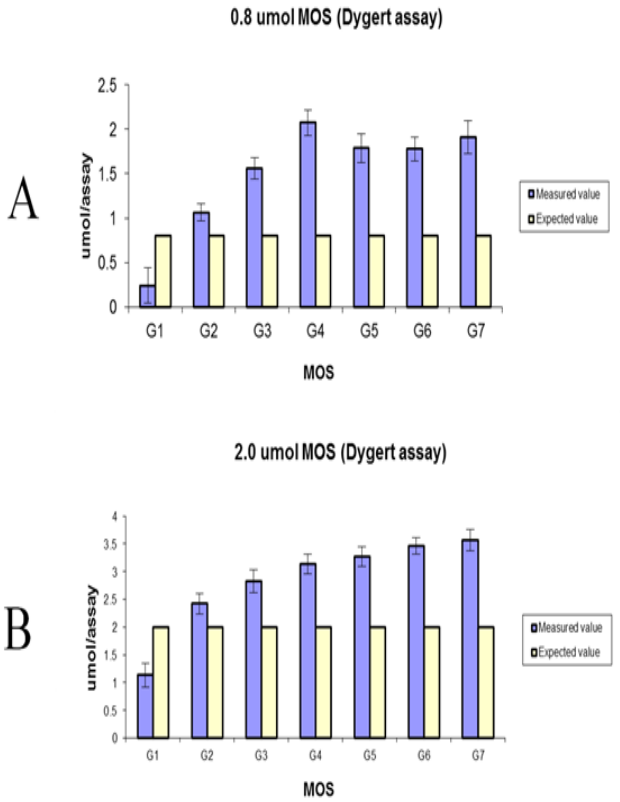

Figure 3. Measured and expected value of different malto-oligosaccharides (MOS) concentration using Dygert assay. A; Measured values compared to the expected value at 0.8 µmol MOS/assay. B; Measured values compared to the expected value at 2.0 µmol MOS/assay. G1 to G7 indicate MOS with one glucose ring (G1) up to MOS with seven glucose rings (G7). Error bars represent ± standard deviations from two duplicates of assay.

measurement to indicate the absence or presence of the reducing sugar rather than for a quantitative measurement of the amount of the reducing sugar in the samples.

For the Dygert assay, the measured values for glucose were only 0.242 µmol and 1.134 µmol compared to the expected values of 0.8 µmol and 2.0 µmol respectively. However, the measured concentrations were higher than the expected values for malto-oligosaccharides with the degree of polymerisation greater than two (Figure 3). For example, at the expected value of 0.8 µmol, maltose was measured at 1.062 µmol increasing to 2.074 µmol for G3. For G5 and G6, the measured values were 1.788 µmol and 1.908 µmol respectively. At the expected value of 2.0 µmol, the Dygert assay showed more or less a linear increase in measured value with increasing degree of polymerisation, with the exception of the one sugar ring, glucose. The observation of a higher measured value than the expected value was probably due to the harsh assay conditions (12 minutes of boiling in alkaline pH) which cleave the α-1,4-linkages therefore creating more reducing ends for the reagent to act upon.Thus, a substrate with a higher degree of polymerisation would have more cleavage of its glucan chain producing an inflated value for the reducing sugar concentrations.

The results of these tests showed that BCA reducing sugar assay was much more accurate than the DNS and Dygert assays. The DNS and Dygert assays overestimated the amount of reducing sugar present as the sugar chain length (degree of polymerisation) increased. Hypothetically, with substrates that have a high degree of polymerisation such as that of amylopectin, DNS and Dygert assays will produce misleading results of debranching enzyme activity by producing very high values for the activity of the enzymes when actual activities were lower due to the overestimation. In addition, DNS and Dygert assays also underestimated the amount of reducing sugar present for glucose. For Dygert assay, comparison between the measured and expected values were conducted at 0.8 and 2.0 µmol due to insensitivity of the reagent at a lower concentration.

The BCA assay is suitable for quantifying reducing sugars even in mixtures of oligosaccharides with different chain lengths. This is especially important in quantifying the enzyme activity of debranching enzymes which cleave substrates releasing a mixture of malto-oligosaccharides of different chain lengths. Thus, in assays of enzymes that debranch sugars with different chain lengths from the same substrate, activities can be measured without the bias towards higher values for enzymes that cleave preferentially the longer chain lengths. With the BCA assay, mixtures of malto-oligosaccharides in the analysed samples can be determined more accurately. This is in contrast with the DNS method, where the response per reducing group is dependent on the length of the oligosaccharide (Doner and Irwin, 1992; Horn and Eijsink, 2004) and cannot accurately quantify a mixture of oligosaccharides. In addition, the BCA reducing sugar assay has been shown to produce highly color-stable properties after the treatment of the reaction mixture with the color-yielding reagents (Utsumi *et al*., 2009).

The BCA assay is also very sensitive compared to the DNS assay. The BCA assay has a sensitivity of 1- 20 nmol/assay (Anthon and Barrett, 2002) while the DNS assay has a sensitivity of about 500 nmol/assay (Moretti and Thorson, 2008). Besides that, the relatively mild condition of the BCA assay provides a method with higher accuracy as there is less degradation of the substrate compared to harsh conditions of both the DNS and Dygert methods (Kongruang *et al.*, 2004).

Conclusions

The BCA assay was the most suitable reducing sugar assay for accurate determination of reducing sugars compared to the DNS and Dygert methods for samples containing mixtures of malto-oligosaccharides of various chain lengths. BCA assay is more sensitive compared to the other two more established methods. Both the DNS and Dygert assays are more suitable for qualitative rather than quantitative measurement of reducing sugars.

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