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Methods for the assay of 1,5-anhydro-D-fructose and α-1,4-glucan lyase

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Abstract

1,5-Anhydro-D-*arabino*-hex-2-ulose (1,5-anhydro-D-fructose, 1,5AnFru), produced by α-1,4-glucan lyase (EC 4.2.2.13) acting on starch, glycogen, or related D-glucose oligo- and polysaccharides as substrate, reacts with alkaline 3,5-dinitrosalicylic acid reagent (DNS) at room temperature (22 °C) within 10 min. The absorbance of the reaction mixture at 550 nm at the end of the reaction was proportional to a 1,5AnFru content in the range of 0.5 to 16 μmol (80 μg to 2.6 mg) mL^{-1}. 1,5AnFru determined by this colorimetric, one test tube one reagent method, was in good agreement with that found by ^{1}H-NMR spectroscopy and HPLC. The DNS method is also specific as other reducing sugars, such as glucose, maltose maltosaccharides, starch and glycogen do not give a colour with DNS at 22 °C; therefore, they do not interfere in the determination. The DNS method is applicable to lyase assay for both cell-free extracts and purified enzyme. Methods for reducing sugar analyses, based on the reduction of ferric and cupric ions, were examined for 1,5AnFru and they proved to be quantitative but in contrast to the DNS method, they were not specific. Instead of assaying 1,5AnFru, the activity of α-1,4-glucan lyase was analysed enzymatically by quantifying glucose or 4-nitrophenol released using maltose and 4-nitrophenyl α-maltopentaoside as substrate, respectively. © 1998 Elsevier Science Ltd.

Keywords: 1,5-Anhydro-D-*arabino*-hex-2-ulose; 1,5-Anhydro-D-fructose; 3,5-Dinitrosalicylic acid; α-1,4-Glucan lyase; Glycogen/starch degradation; Sugar analysis

Abbreviations: 1,5AnFru: 1,5-Anhydro-D-*arabino*-hex-2-ulose (1,5-anhydro-D-fructose); BCA: 4,4′-Dicarboxy-2,2′-biquinoline (2,2′-bicinchoninic acid); DNS: 2-Hydroxy-3,5-dinitrobenzoic acid (3,5-dinitrosalicylic acid); G-6-PDH: Glucose 6-phosphate dehydrogenase; GOD: Glucose oxidase; HK: Hexokinase; Mops: 4-Morphorlinepropanesulfonic acid; G_5-NP: 4-Nitrophenyl α-maltopentaoside; POD: Peroxidase

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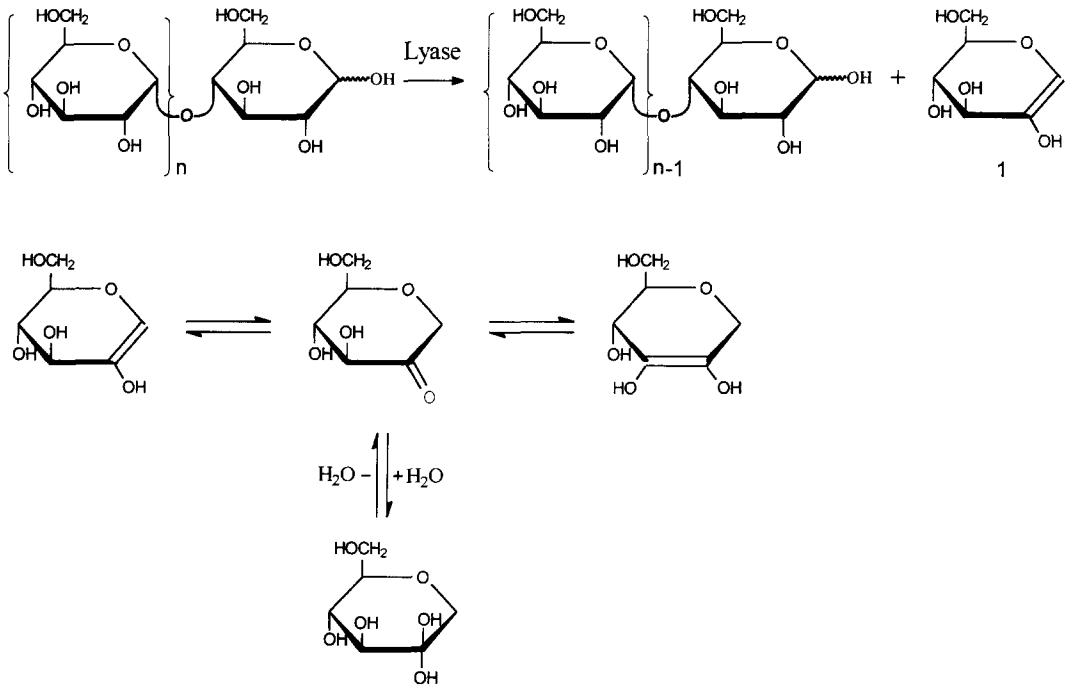
1. Introduction

α-1,4-Glucan lyase (EC 4.2.2.13) catalyses the degradation of α-glucans and produces 1,5-anhydro-D-*arabino*-hex-2-ulose (1,5-anhydro-D-fructose, 1,5AnFru) (Scheme 1). The formed 2-enol form of 1,5AnFru is in an equilibrium with a 2,3-enediol form, a keto form and the hydrated form. The latter is the dominating species in water [1–3]. The substrate can be maltose, maltosaccharides, amylose, amylopectin or glycogen. When a linear α-(1 → 4)-glucan is used as substrate, besides 1,5AnFru, D-glucose is also produced as the degradation starts from the non-reducing end of the α-glucan molecule until the last glucose unit is left. When a branched glucan, such as amylopectin and glycogen, is used as substrate, the products are 1,5AnFru and limit dextrin, as the lyase stops its lytic action when a branching point of α-(1 → 6)-linkage is met [3]. The α-1,4-glucan lyase has been purified and cloned [1,4]; this makes a scale-up production of 1,5AnFru by enzymatic method possible.

1,5-Anhydro-D-fructose was first prepared through chemical synthesis in 1980 [5], and later it was found to occur also naturally [6]. As the synthesis of 1,5AnFru involves multiple steps with low yields [5], there has been limited availability of 1,5AnFru. So, very little work has been done on 1,5AnFru concerning its chemistry, metabolic role and its possible uses in medicine. It is only known at present that 1,5AnFru is a precursor for antibiotics formation in fungi [7] and that 1,5AnFru is reduced to 1,5-anhydro-D-glucitol, for example, in erythroleukemia cells [8]; 1,5-anhydro-D-glucitol is used as a diagnostic marker for diabetes [9]. In red seaweeds [3], fungi [6,7] and probably in many other organisms including humans [8], the degradation of glycogen/starch via 1,5AnFru obviously represents a new, alternative degrading route other than those initiated by phosphorylase and hydrolases such as amylases [10].

Analysis of 1,5AnFru has been performed previously by NMR spectroscopy, GC–MS, or HPLC following chemical derivatisation [6,8]. These methods are tedious for routine assay of 1,5AnFru and lyase activity. To facilitate research in this area, simple, fast and specific methods are needed for the assay of 1,5AnFru and lyase.

Analysis of sugars was an important part of biochemistry in the early part of this century. The interest has been renewed in the last decade due to the wide use of reducing sugar releasing enzymes and the fast advance of glycobiology. Hence, the methods for sugar analysis are numerous [11–24]. There are still continued efforts in improving these methods for accuracy and fast sample handling [19–21]. The principles of these chemical methods are based mostly on



Scheme 1. Formation of the enol form of 1,5AnFru by the action of α-1,4-glucan lyase on α-(1 → 4)-glucan, and the keto-enol tautomerization and the hydration of the keto form of 1,5AnFru.

the capability of sugars to reduce ferric, cupric ions or organic reagents [11,12,14]. The improvements of these methods are largely on the choice of new chelating agents for stabilizing the transition-metal ions or on the choice of reagents that can develop colour with ferrous or cuprous ions formed [12,13,19,23]. Heating at 100 °C for 5 or up to 30 min, depending on the sugar species, is often necessary to complete these reactions [24]. Furthermore, none of these colorimetric methods is specific for a given sugar; therefore, the total reducing sugars in a sample are measured. Because of this unspecificity, it has always been problematic to adapt these methods to the assay of reducing sugar releasing enzymes [25,26], since the activity measured represents the sum of all reducing sugar producing enzymes in the assay system [9,25]. As a result, the identity of the enzyme studied will only be known when the reaction pattern is examined using the finally purified enzyme [27–29].

Enzymatic methods developed for sugar analysis are specific [30], but for the assay of reducing sugar releasing enzymes, these methods obviously suffer from the interference of other sugar metabolizing enzymes in the assay system. To overcome these problems, special substrates such as non-reducing end blocked 4-nitrophenyl α-maltoheptaoside and 4-nitrophenyl α-maltopentaoside have been designed for the specific assay of alpha-amylase and beta-amylase, respectively [25,26,31–33]. The pitfalls are, however, that the reactivity and kinetics of alpha- and beta-amylases on these low molecular weight substrates can be different from those with high molecular weight substrates such as amylose and amylopectin [25].

In the present report, several colorimetric and instrumental methods are evaluated for the analysis of 1,5AnFru and for the assay of the lyase activity with respect to both specificity and sensitivity. One of them, the DNS method, was examined in detail and found specific for 1,5AnFru and lyase activity analyses relative to other reducing sugars and hydrolases. This makes α-1,4-glucan lyase one of the few enzymes that can be assayed specifically among the huge group of polysaccharide degrading enzymes.

2. Results and discussion

Reaction of 1,5AnFru with DNS reagent.—The reducing capability of 1,5AnFru was tested in the presence of the DNS reagent. It was found that

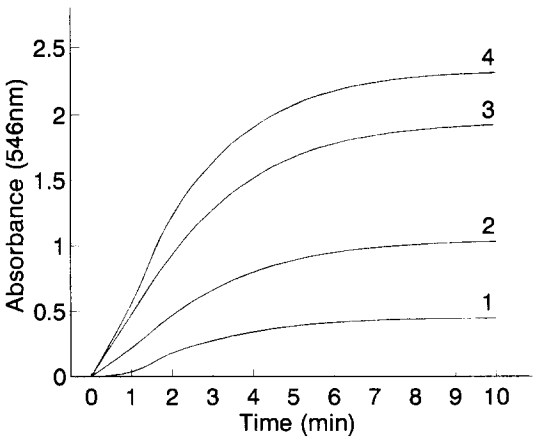


Fig. 1. Time-course of 1,5AnFru reaction with the DNS reagent at 22 °C as monitored at 546 nm. The reaction was started by adding 0.3 mL of the DNS reagent to 0.3 mL of 1,5AnFru sample containing 0.55 (1), 1.26 (2), 2.34 (3), and 2.84 (4) μmol of 1,5AnFru, respectively.

1,5AnFru exhibited extraordinarily high reducing power, as the reaction of 1,5AnFru with the DNS reagent was completed in less than 10 min at room temperature (22 °C). The reaction progress of varying amounts of 1,5AnFru with the DNS reagent, as monitored at 546 nm, is shown in Fig. 1. The presence of organic solvents, such as dimethylsulfoxide, further shortened the 1,5AnFru–DNS reaction time (data not shown). The fast reaction of 1,5AnFru with the alkaline DNS reagent is thus in sharp contrast with reducing sugars, such as D-glucose and maltose, which is completed only at 100 °C for 5–10 min [15,30].

For the 1,5AnFru–DNS reaction, the same maximal absorbance at 546 nm was reached, regardless of whether the reaction was carried out at room temperature for 10 min or at 100 °C for 2–5 min. After full development of the colour at 22 °C in 10 min, the 1,5AnFru–DNS reaction solution began to fade slowly, and after 40 min, it decreased by about 5%. For quantitative assay of 1,5AnFru, it is recommended that the absorbance of the 1,5AnFru–DNS solution be measured at 10 min after the 1,5AnFru sample and the reagent is mixed. Another advantage to keep the reaction time short is the hindrance of interference from other reducing sugars as, at longer reaction time, common reducing sugars will also slowly react with the DNS reagent (see below).

The absorbance spectra of the 1,5AnFru–DNS reaction mixture were similar to those with D-glucose (not shown). From the absorbance spectrum, a wavelength at 546 nm was chosen for the 1,5AnFru assay, the same as adopted for reducing sugars [10], and

when using microplate readers, to be close to this wavelength, a filter of 550 nm was chosen.

The following monosaccharides and maltosaccharides were tested for their reactivity with the DNS reagent at room temperature. It was found that the reaction was slow, as the absorbance ratio of 10 mM of each of these saccharides to 10 mM of 1,5AnFru at 550 nm was zero and around 2% after their reaction with the DNS reagent for 10 and 40 min, respectively. The sugars examined were: D-xylose, D-ribose, D-arabinose, D-fructose, D-mannose, D-galactose, L-rhamnose, D-glucuronic acid, 2-amino-2-deoxy-D-glucose, 2-deoxy-D-glucose, D-glucose, maltose, maltotriose, maltotetraose, maltopentaose and maltohexaose. Most of these sugars are the products of hydrolases acting on glycosides, oligo- and polysaccharides. The malto-oligosaccharides are also the reaction intermediates when linear malto-oligosaccharides and glucans are used as substrate [3]. Substrate levels of maltose, amylopectin and glycogen did not interfere with the 1,5AnFru–DNS reaction. Only in the presence of extremely high concentrations of reducing sugars can the 1,5AnFru content be overestimated. For instance, 1,5AnFru was overestimated by 14% in the presence of 0.5 M glucose.

Under the 1,5AnFru assay conditions, the reductants dithiothreitol (0.1 M) and β-mercaptoethanol (1 M), usually used to protect enzymes, were inactive toward the DNS reagent. Common buffers, salts, and ethanol (up to 30%, v/v) used in the enzyme assays and 1,5AnFru preparation did not affect the reaction of 1,5AnFru with the DNS reagent. The buffers and salts tested were acetate, phosphate, Mops, *N*,*N*-bis(2-hydroxyethyl)glycine (Bicine), citrate, 1,3-bis[tris(hydroxymethyl)methylamino]propane (Bis-tris-propane), sodium azide, and sodium chloride.

The reaction mechanism of 1,5AnFru under alkaline conditions and in the presence of DNS has been examined [35]. It seemed that 1,5AnFru is oxidised to 1′,4-anhydro-2-*C*-(hydroxymethyl)-D-arabonic acid while 3,5-dinitrosalicylic acid is reduced to 3-amino-5-nitrosalicylic acid (3-amino-2-hydroxy-5-nitrobenzoic acid) as has been proposed for reducing sugars [14].

As a hypothetical explanation to the high reactivity of 1,5AnFru with DNS reagent as opposed to other reducing sugars, 1,5AnFru possesses the unique structure of a carbonyl group that does not form any hemiacetal bonding as other reducing sugars do. 1,5-Anhydro-D-fructose exists in aqueous solution in an equilibrium mixture among the 2,3-enediol form, 2-enol form, keto-, and hydrated forms (Scheme 1), with the hydrated form as the dominating species [3,34]. The enediol form of 1,5AnFru is supposed to be the most reactive species. The formation of the enediol form of 1,5AnFru in alkaline conditions may therefore be easier than other reducing sugars, whose open-chain forms have to be produced by the breakage of a hemiacetal or hemiketal linkage before a 1,2-enediol intermediate can be formed. By NMR spectroscopy, it was confirmed that the enediol form of 1,5AnFru was the dominating species in alkaline aqueous solution. The even faster reaction of 1,5AnFru with the alkaline DNS reagent in organic solvents may further support that it is the enediol form of 1,5AnFru that is most active since these solvents favour the formation of enediol/keto-form of 1,5AnFru instead of the hydrated form of 1,5AnFru.

1,5-Anhydro-D-fructose standard curves.—1,5AnFru standards were prepared by a complete degradation of known amounts of [U-^{14}C]maltose. Like the unlabelled maltose [3], the product ratio of 1,5AnFru to glucose for [U-^{14}C]maltose was 1, as confirmed by electronic autoradiography which measures the radioactivity of each spot as counts per minute after separation on TLC. The amount of glucose was further confirmed by HPLC. The amount of 1,5AnFru produced was thus deduced from the amount of maltose or glucose formed. Using these [U-^{14}C]1,5AnFru samples, a standard curve was made by reaction with the DNS reagent (Fig. 2). The absorbance (0.2 to 2.6 at 550 nm) as measured using microplate reader was linear for a 1,5AnFru range of 2 to 18 μmol (0.3 to 3.0 mg) mL^{-1} with a correlation coefficient of 99.9% (Fig. 2).

From Fig. 2, a formula with $y = ax + b$ was obtained, where y is 1,5AnFru in μmol and x the absorbance at 550 nm, a and b are coefficients. After 5 repeated experiments, a was calculated to be 0.696 and b 0.058. The coefficient of variation for a was less than 1.5% and that for b was as high as 15%; therefore, for accurate analysis, especially at lower 1,5AnFru concentrations, the samples were assayed each time together with standards.

From Fig. 2, the 1,5AnFru content could not be correctly estimated when it falls below 0.2 μmol. This was overcome by adding 0.2 μmol 1,5AnFru to both blanks and 1,5AnFru samples and, in this way, a linear curve that passed the point zero was obtained (not shown). By this improvement, the 1,5AnFru assay range was from 0.5 to 16 μmol (80 μg to 2.6 mg) mL^{-1}. It is known that dissolved molecular oxygen interferes with reducing sugar assays and this

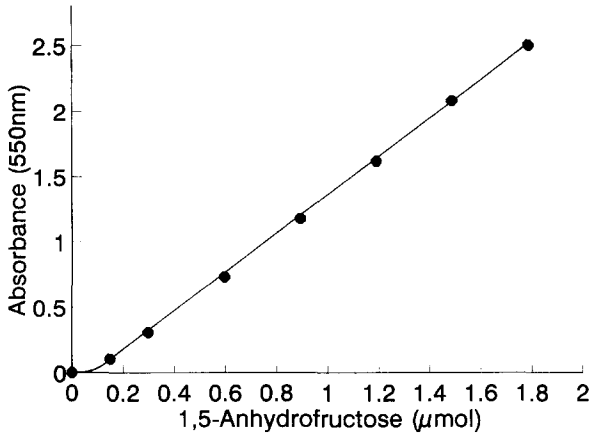


Fig. 2. The standard curve of 1,5AnFru produced using the DNS method. The 1,5AnFru sample (0.1 mL) containing varying amounts of [U-^{14}C]1,5AnFru as indicated was reacted with 0.1 mL of DNS reagent. After 10 min at 22 °C, the reaction mixture was analyzed at 550 nm using a microplate reader. The 1,5AnFru sample was obtained after complete degradation of [U-^{14}C]maltose by the algal lyase that produced equal molar amounts of 1,5AnFru and D-glucose.

could be overcome either by purging the assay solutions with inert gas prior to the assay or by adding fixed, known amount of reducing sugar to all the samples and blanks [36].

To test the reliability of the DNS method, one sample with a 1,5AnFru concentration of 11.08 mM was assayed 33 times over a period of 5 months. The average absorbance at 550 nm was 1.444 with a standard deviation of 0.079.

Similar results were obtained when the 1,5AnFru–DNS reaction was monitored at 546 nm using a spectrophotometer with cuvettes of 1 cm light path (not shown). In this case, the absorbance from 0.1 to 2.3 was linear to a 1,5AnFru content in the range of 1 to 9 μmol (0.16 to 1.46 mg) mL^{-1}.

Due to its rapidity and simplicity, the DNS reagent has persisted in use for measuring of general reducing sugars and the method has been automated [17,18], but just as the other methods for reducing sugar analysis, it is unspecific and heating at 100 °C in a water bath for 5 to 10 min is needed. Besides, the reducing efficiency varies with different sugars [17,18] and equimolar quantities of maltodextrins give absorbance values increasing with the number of D-glucose units in the dextrin [15]. Because of this the DNS method is not suitable for accurate analysis of total reducing sugars and for the assay of reducing sugar releasing enzymes like alpha-amylases, whose products are a mixture of D-glucose and maltodextrins with changing polymerization degrees during the course of amylolytic reaction [15]. For the assay of 1,5AnFru using the DNS reagent, such is not a problem, since all the other reducing sugars and maltodextrins will simply not react with the DNS reagent at room temperature in 10 min. This forms the basis for the specific analysis of 1,5AnFru. The application of the DNS method in assay of purified fungal lyase and of algal lyase in cell-free extract, as expressed in a time course release of 1,5AnFru from the substrate glycogen and maltose is shown in Figs. 3 and 4, respectively.

Determination of 1,5-anhydro-D-fructose by other reducing sugar assay methods.—In principle, pure 1,5AnFru can be analysed by all the colorimetric methods for reducing sugar analysis. Examples are the alkaline ferricyanide method based on the reduction of ferric ions, the Somogyi–Nelson and the BCA methods based on the reduction of cupric ions. Common to these three methods is that the reaction with 1,5AnFru has to be carried out at 100 °C; therefore, other reducing sugars present will also react. For example, at room temperature, 1,5AnFru reduced ferric ions faster than any other reducing sugars, but a complete oxidation of 50 nmol of 1,5AnFru took 1 h. If heated at 100 °C, the reaction was completed in 5 min. Thus, for the ferricyanide method [13], the loss of absorbance at 420 nm was linear for a 1,5AnFru

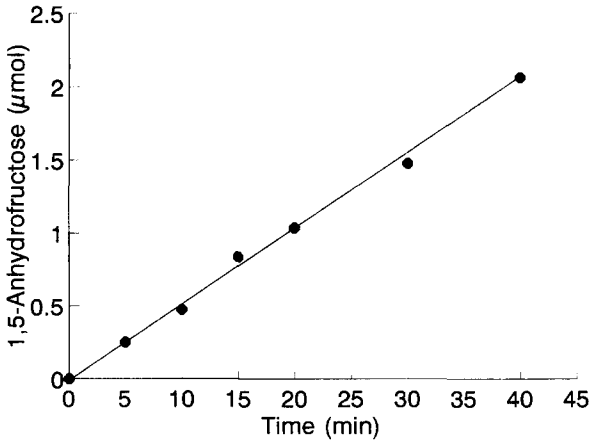


Fig. 3. Time-course release of 1,5AnFru from glycogen by the fungal lyase at 48 °C as monitored using the DNS method. The reaction mixture contained glycogen (20 mg mL^{-1}) and the lyase from *M. vulgaris* (0.44 μg) in Mops–NaOH (20 mM, pH 6.5). At the time interval indicated, 0.1 mL reaction mixture was taken and heated at 100 °C for 2 min and then kept on ice before all the samples were ready for the addition of 0.1 mL DNS reagent for analysis of 1,5AnFru.

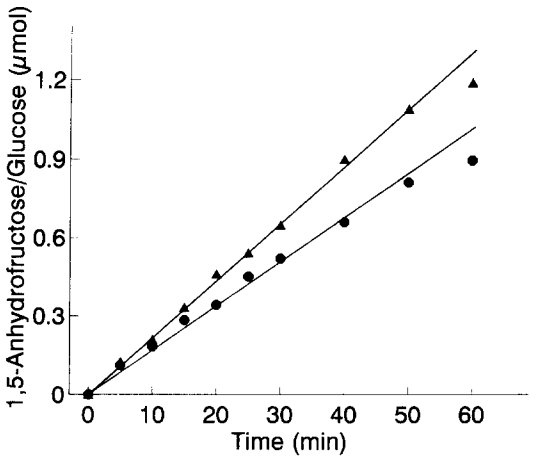


Fig. 4. Time-course release of 1,5AnFru and glucose from maltose using the algal cell-free extract as a lyase source. The reaction mixture in a final volume of 0.3 mL contained 1.2 mg maltose in 50 mM Mops–NaOH (pH 6.2), and 50 μL cell-free extract made in the same buffer. The reaction was carried out at 30 °C for varying time as indicated and was stopped by heating at 100 °C for 2 min. 1,5AnFru and D-glucose were assayed by the DNS method. (●) and the GOD/POD method (▲), respectively. For the glucose assay by the GOD/POD method the samples were diluted by a factor 6.7.

content of 0.12–1.18 μmol (19–190 μg) mL^{-1}, when the reaction was performed at 100 °C for 5 min. The ferricyanide method can therefore be used for both 1,5AnFru analysis and for the assay of lyase activity, provided that other reducing sugars are absent. For the assay of lyase activity, amylopectin instead of maltose is the preferred substrate.

By the Somogyi–Nelson copper reduction method, the absorbance at 620 nm was linear in the range of 0.3–3.0 μmol (49–486 μg) mL^{-1}, when the reaction was carried out at 100 °C for 2 min. As the ferricyanide method for the lyase assay, amylopectin instead of maltose is the preferred substrate and the assay system has to be free from starch hydrolases.

By the BCA method, The absorbance at 550 nm was linear to a 1,5AnFru content of 13–67 nmol (2–11 μg) mL^{-1}, when the reaction was performed at 100 °C for 5 min. The sensitivity of the BCA method is thus 10 times more than that of the other photometric methods examined above. It was, however, only suitable for the quantification of pure 1,5AnFru samples, not suitable for assay of lyase activity using cell-free extracts; the presence of substrate usually produced high background with the BCA reagent when heated.

Being a keto-sugar, 1,5AnFru could also be analysed by the anthrone method specific for ketoses [22]. Again, it proved that the reaction had to be carried out at elevated temperatures, for example at 55 °C for 90 min. At such conditions, however, fructose, fructose 1,6-bisphosphate, sucrose and inulin will also react [22].

Non - colorimetric analysis of 1, 5 - anhydro - D - fructose.—To validate the DNS method, a 1,5AnFru sample prepared from amylopectin was analysed by ^{1}H-NMR spectroscopy and subsequently by the DNS method. For the quantification of 1,5AnFru using the ^{1}H-NMR method, freeze-dried 1,5AnFru samples were dissolved in deuterium oxide with *N,N*-dimethylformamide added as an internal standard. After NMR spectroscopy analysis, the 1,5AnFru samples were assayed by the DNS method. A good agreement between the 1,5AnFru determined by the ^{1}H-NMR method and the DNS method is shown in Table 1. The presence of dimethylformamide did not interfere with the 1,5AnFru–DNS reaction.

1,5-Anhydro-D-fructose was analysed on HPLC with a carbohydrate column and a differential refractometer, using water as the eluent. The response of 1,5AnFru was linear in a 1,5AnFru range of 1–384 μmol (0.16–62.26 mg) mL^{-1} in an injection volume of 50 μL. When analysed by HPLC on a Dionex anion exchange chromatography system equipped with a pulsed amperometric detection, the linear range for 1,5AnFru was 12.5–250 nmol (2.0–40.5 μg) mL^{-1} in an injection volume of 20 μL.

Table 1
A comparison of 1,5AnFru samples quantified by ^{1}H-NMR spectroscopy and by the DNS method

Methods	Medium used	Average (mg)	Number of samples	SD[a]
^{1}H-NMR method	Deuterium oxide	35.000	6	0.063
DNS method	Deuterium oxide	35.432	6	1.498
DNS method	Water	35.393	4	0.422

Freeze-dried samples containing equal amount of 1,5AnFru were dissolved either in water or D_2O oxide with DMF added as an internal standard. The 1,5AnFru samples in deuterium oxide and DMF were first analyzed by ^{1}H-NMR spectroscopy and all the samples were subsequently analyzed by the DNS method.
[a]Standard deviation.

The limitations of these instrumental methods are their requirement of pure or relatively pure 1,5AnFru samples, in the case of ^{1}H-NMR spectroscopy and HPLC, respectively.

Assay of α-1,4-glucan lyase.—The activity of α-1,4-glucan lyase was measured by the assay of 1,5AnFru released as discussed above. The lyase activity was also assayed using ^{14}C-labelled maltose or ^{14}C-labelled starch as substrate and detecting the radioactive 1,5AnFru formed by electronic autoradiography after separation on TLC plates (not shown). Alternatively, the lyase activity was measured enzymatically by the assay of D-glucose or 4-nitrophenol released. The methods measuring glucose are based on the observation that maltose is degraded by α-1,4-glucan lyases to produce 1 mol of glucose and 1 mol of 1,5AnFru [3]. The glucose produced was determined by using the HK/G-6-PDH method and the GOD/POD method. By the HK/G-6-PDH method, the production of NADPH, as indicated by the increase in absorbance at 340 nm, was proportional to the amount of glucose released in the range of 67–333 nmol (11–54 μg) mL^{-1}. By the GOD/POD method, linear assay range was 33–367 nmol (5.4–59.5 μg) mL^{-1}. These two methods are thus about 5 times more sensitive than the DNS method. Under the assay conditions, the 1,5AnFru produced did not interfere with the tool enzymes used in the glucose assays, since in the presence of 1,5AnFru the glucose measured was in good agreement with that measured by HPLC. For instance, two samples measured by the GOD/POD method were 13.3, and 32.6 μmol in 1 mL reaction mixture, while the corresponding values were 14.1, and 31.7 μmol, as determined on HPLC with a carbohydrate column and a differential refractometer.

When the algal cell-free extract was used as the lyase source, the release of 1,5AnFru and glucose from maltose was linear up to 50 min (Fig. 4). It can be seen that, under the assay conditions, the lyase activity was somewhat overestimated by assaying the glucose released than by that of 1,5AnFru (Fig. 4) at a longer incubation time. This could be due to interfering enzymes in the cell-free extract.

These two enzymatic methods are, however, not suitable when longer maltosaccharides are used as substrates, as the ratio of glucose to 1,5AnFru decreases with the increase in DP number of an α-(1 → 4)-glucan molecule [3]. Extra high amount of 1,5AnFru could be a potential inhibitor for the tool enzymes used in the assay [34]. Also, the lyases from different sources vary with the reactivity to maltose and the fungal lyase shows less than 10% activity toward maltose compared to maltotetraose [37]. The enzymatic methods are not applicable to amylopectin or glycogen as substrate, as in these cases, the enzyme products are 1,5AnFru and limit dextrin [3]. It is clear that for the HK/G-6-PDH and the GOD/POD methods, the assay system should be free of D-glucose-metabolizing enzymes.

The third enzymatic method is based on the fact that the lyase degrades G_5-NP to 1,5AnFru and 4-nitrophenol as the final products. The rate decreased gradually as G_5-NP was reduced to lower 4-nitrophenyl α-maltooligosaccharides; hence, an α-glucosidase was included in the assay to promote the release of 4-nitrophenol. This method was originally designed for the assay of beta-amylase [24,31,32] and it proved also suitable for the assay of α-1,4-glucan lyases from both fungal and algal sources in this study, as the release of 4-nitrophenol was linear with the time progress (Fig. 5) and the amount of lyase present (not shown). The presence of 1,5AnFru neither inhibited the α-glucosidase nor affected the colour development of 4-nitrophenol at alkaline conditions. When using the G_5-NP method, the system should obviously be free from beta-amylase.

The activities determined using maltose and G_5-NP as substrates may not reflect the in vivo starch/glycogen degrading activity by the lyase as they do not represent the major naturally occurring substrates, but

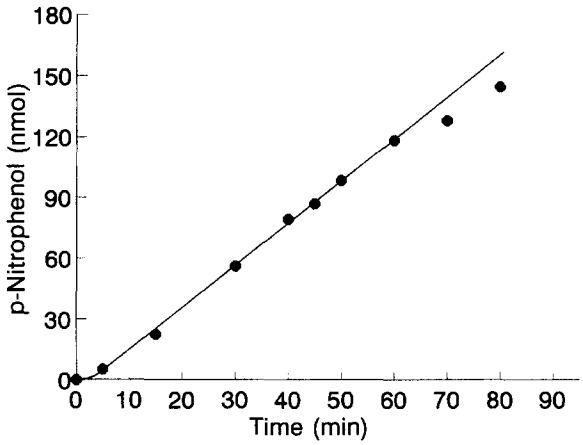


Fig. 5. Time course release of 4-nitrophenol from 4-nitrophenyl α-maltopentaoside (G_5-NP) by the fungal lyase. The reaction mixture contained G_5-NP (2.5 μmol), α-glucosidase (50 units), Mops-NaOH (20 μmol, pH 6.2) and the fungal lyase of *M. costata* (5.32 μg protein) in a final volume of 1 mL. The reaction was carried out at 40 °C and at the time intervals indicated, aliquots of 0.1 mL were taken for analysis of released 4-nitrophenol.

they are methods of choice for quantifying the lyase activity and for characterizing purified lyases.

It is concluded that assay of 1,5AnFru by the DNS method is both quantitative and specific relative to the other reducing sugars. Among all the methods examined, this method is a basic one. To check, for example, the occurrence of lyase activity in the cell-free extracts and to verify whether the enzyme is a lyase or a common reducing sugar-releasing enzyme (amylases, etc.), the DNS method could not be replaced with any other reducing sugar analysis methods. Actually, by using the DNS method, we have demonstrated the existence of and purified α-1,4-glucan lyases from red seaweeds [1] and fungi [37]. The DNS method also proved to be very useful in identifying positive clones of the transgenic *Aspergillus niger* and *Pichia pastoris* that produced the algal and fungal α-1,4-glucan lyases (Yu and Marcussen, unpublished data).

3. Experimental

Materials and chemicals.—The marine red alga *Gracilariopsis lemaneiformis* was collected from Qingdao (China). The fungi *Morchella costata* (ATCC 64173) and *M. vulgaris* (ATCC 64176) were obtained from American Type Culture Collection (ATCC, Rockville, Maryland). They were grown at 25 °C on a shaker using the culture medium recommended by ATCC. The α-1,4-glucan lyases were purified to homogeneity from the red alga and the fungi [1,37]. Cell-free extracts of this red alga and the two fungi were made in 50 mM Mops–NaOH (pH 6.2) as described earlier [1,37]. D-Glucose was from BDH (Poole, England). Maltose was from Fluka (Buchs, Switzerland). [U-^{14}C]Maltose was from Amersham Life Science (Little Chalfont, UK). HK/G-6-PDH mixture was from Boehringer Mannheim (Mannheim, Germany). An assay kit for D-glucose using GOD/POD was bought from E. Merck (Darmstadt, Germany). An assay kit for beta-amylase containing G_5-NP and α-glucosidase was from MegaZyme (Aust) Pty. (Sydney, Australia). Disodium 2,2′-bicinchoninate, 4-nitrophenol standard solution (10 μmol mL^{-1}), [U-^{14}C]starch, 3,5-dinitrosalicylic acid and all other chemicals were purchased from Sigma (St. Louis, USA).

Assay of 1, 5AnFru by the DNS method.—The DNS reagent was prepared according to Steup [10]. It was made by dissolving 1 g of 3,5-dinitrosalicylic acid in 1 N NaOH (40 mL) and water (30 mL) at elevated temperature. To this soln, potassium sodium tartrate (3 g) was added and dissolved. After cooling to 22 °C, the soln was diluted to a final volume of 100 mL and kept in an air-tight brown bottle before use. The reagent was stable for several months. 1,5AnFru samples were diluted to the range of 0.2 to 1.8 μmol per 0.1 mL followed by the addition of an equal vol of DNS reagent (0.1 mL). The reading at 550 nm was monitored using a microplate reader (Model EAR 340 AT, SLT-Labinstruments, Grödig, Austria) after standing at room temperature for 10 min. Alternatively, the reaction of 1,5AnFru (0.3 mL) with the DNS reagent (0.3 mL) was monitored at 546 nm using a Hitachi UV-2000 double beam spectrophotometer (Tokyo, Japan). It was calibrated with respect to wavelength, spectrum bandwidth and baseline flatness according to manufacturer's instructions before use.

Assay of 1,5AnFru by other reducing sugar assay methods.—The analyses of 1,5AnFru by the alkaline ferricyanide, the Somogyi–Nelson and the BCA methods were performed as described earlier for the assay of common reducing sugars [11,38,19], except that the reaction time at 100 °C was shortened to 2–5 min. The ferricyanide reagent contained 4.3 mM potassium ferricyanide and 283 mM sodium carbonate [11]. To this reagent (325 μL) was added the 1,5AnFru sample (50–500 nmol) and water to a final volume of 750 μL and then mixed and heated at 100 °C for 5 min. After cooling, absorbance at 420 nm was measured against a blank. For the Somogyi–Nelson method, the 1,5AnFru sample (0.1 mL, 30–300 nmol) was heated with the Somogyi copper soln (0.2 mL) at 100 °C for 2 min. After cooling, the Nelson soln (0.2 mL) was added. After shaking and addition of water (1 mL), aliquots of 0.2 mL were used for measurement of absorbance at 620 nm using the microplate reader.

The BCA reagent was prepared as described earlier [19,20]. Solution A contained BCA, Na_2CO_3 and $NaHCO_3$; solution B contained copper sulfate and L-serine. The BCA reagent was a mixture of equal vols of soln A and B. To 1,5AnFru sample (0.15 mL, 2–10 nmol) was added the BCA reagent (0.15 mL) and the mixture was heated at 100 °C for 5 min. After cooling, an aliquot of 0.2 mL was used for microplate analysis and the absorbance at 550 nm was determined against a blank.

The assay of 1,5AnFru using the anthrone–tryptophan–sulfuric acid reagent was done according to Somani et al. [22] and the absorbance of the 1,5AnFru reaction mixture was monitored at 512 nm.

Non-colorimetric analyses of 1,5AnFru.—In the NMR spectroscopy quantification of 1,5AnFru, ^{1}H-NMR spectra were recorded in D_2O on a Bruker AC250P instrument by adding a known amount of DMF as internal standard. The signals of 1,5AnFru-protons were integrated over the range 3.3–4.0 ppm vs. the two DMF methyl group signals at 2.8 and 3.0 ppm. Careful baseline correction was performed using the standard integration routine. The accuracy of the procedure was examined on dried and weighed sucrose using DMF as internal standard. Integration of the range 3.3–4.3 ppm (representing the 13 protons of sucrose) vs. the methyl groups of DMF gave an accuracy of $\pm 1\%$ in three independent experiments.

For HPLC determination of 1,5AnFru and glucose, the enzyme reaction mixture was analysed on a Waters HPLC instrument (model WISP 710B) equipped with a differential refractometer (model 410). The column used was a carbohydrate Ca^{2+} column (30 × 0.65 cm, Chrompack, Middelburg, The Netherlands); the injection vol was 50 μL containing 1,5AnFru from 50 nmol to 19.2 μmol. The analysis of 1,5AnFru on a Dionex system was as described earlier [3] and the injection vol used was 20 μL containing 1,5AnFru from 0.25 to 5 nmol.

The quantification of ^{14}C-labelled 1,5AnFru, D-glucose and maltose on TLC plates as their cpm values was performed by electronic autoradiography using an Instant Imager™ instrument (Packard Instrument, Meriden, CT). The separation of 1,5AnFru, D-glucose and maltosaccharides by TLC on silica gels was achieved as described earlier [3].

Assay of α-1,4-glucan lyase.—For the assay of α-1,4-glucan lyase, the standard assay mixture consisted of maltose, amylopectin or glycogen in 50 mM Mops–NaOH (pH 6.2). For the determination of 1,5AnFru produced at the end of the reaction, to the reaction mixture was added an equal vol of the DNS reagent and assayed for 1,5AnFru as above. For the determination of glucose released by the HK/G-6-PDH or GOD/POD methods, the reaction was stopped by heating at 100 °C for 2 min at the end of the reaction. In the HK/G-6-PDH method, to 0.3 mL reaction mixture (containing glucose 20–100 nmol) was added 0.5 mL of Tris–HCl (160 mM, pH 7.5) containing $MgCl_2$ (20 mM), 50 μL of ATP (9 mM), 50 μL of NADP (12 mM), HK (0.8 units), and G-6-PDH (0.4 units) in a final volume of 1.0 mL. The absorbance was monitored at 340 nm after incubation at 30 °C for 10 min. Typically, 0.1 μmol of glucose gave a reading of 0.580 at 340 nm. In the GOD/POD method, to 0.3 mL heated reaction mixture (containing glucose 10–110 nmol), was added 1 mL reagent containing GOD (6 units), POD (3.2 units), mutarotase (0.05 units), 4-amino-antipyrine (1 μmol), 4-hydroxybenzoic acid (6 μmol) in 1,4-piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes, 50 mM, pH 7.2). After incubation at 30 °C for 30 min, the absorbance at 500 nm was monitored. Typically, 0.1 μmol of glucose gave a reading of 0.480 under these conditions.

For the assay of the lyase activity with G_5-NP as substrate, to 0.5 mL of the substrate solution containing G_5-NP (2.5 μmol) and α-glucosidase (50 units) was added 0.4 mL of Mops–NaOH (50 mM, pH 6.2) and the lyase in a final volume of 1 mL. The reaction was carried out at 40 °C and at certain time intervals, an aliquot of 0.1 mL was taken for analysis by adding 0.75 mL of 1% (w/v) trizma base. Aliquots of 0.25 mL were used for analysis of the released 4-nitrophenol at 410 nm by using the microplate reader.

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