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MEASUREMENT OF INULIN AND INULIN-DEGRADING ENZYMES

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ABSTRACT
A non-instrumental method for the measurement of fructan is described. The method simplifies fructan analysis, is easy to perform, uses standard laboratory equipment, and is accurate, reproducible and specific. The procedure employs highly purified and specific enzymes to hydrolyse sucrose, starch and fructans (inulins and graminan).

1 INTRODUCTION

Fructans are defined as any compound where one or more fructosyl-fructose linkages constitute a majority of the linkages (Lewis, 1993). This refers to polymeric material as well as oligomers as small as the disaccharide, inulobiose. Material included in this definition may or may not contain attached glucose. The terms oligomer and polymer are used by fructan researchers to distinguish between materials which can be specifically characterized and those which cannot.

Fructans are widely distributed in the plant kingdom. They are present in monocotyledons, dicotyledons and in green algae (Pontis, 1990).They differ in molecular structure and in molecular weight. They may be classified in three main types (Pontis, 1990; Waterhouse and Chatterton, 1993): the inulin type, the levan type and the graminan type. The inulin type consists of material that has mostly or exclusively the (2→1) fructosyl-fructose linkage. Levan is material which contains mostly or exclusively the (2→6) fructosyl-fructose linkage. The graminan type has both (2→1) and (2→6) fructosyl-fructose linkages in significant amounts (*e.g.* graminan from Gramineae). In the context of this paper, the term fructan will relate just to inulin and graminan. The analytical procedure for the measurement of fructan reported here, has not been evaluated on levan.

Several procedures have been described for the measurement of fructan in plant material and food products. It is generally accepted that these are best measured after hydrolysis to fructose (and glucose). This introduces the problem of independently removing, or measuring, sucrose, fructose and glucose. Pontis (1966) has reported the removal of sucrose, glucose and fructose by hydrolysing sucrose with a crystalline yeast invertase and destroying the resulting glucose and fructose as well as existing monosaccharides by boiling with sodium hydroxide. It was claimed that the action of invertase on the lower fructan members of the inulin series is slow and can be rendered insignificant by judicious selection of the incubation conditions. However, experimental data was not provided. In testing currently available pure yeast invertases from baker's yeast (Sigma cat. no. I-4504) and *Candida utilis* (Sigma cat. no. I-4753), we have found that it is extremely difficult, if not impossible to achieve these conditions, as shown in Fig. 1. In this figure, the relative rates of hydrolysis of sucrose, 1-kestose, 1,1-kestotetraose, 1,1,1-kestopentaose and Jerusalem artichoke inulin (polysaccharide fraction) by yeast invertase are compared. It is evident that 1-kestose is hydrolysed at approximately 20% the rate for sucrose, and 1,1-kestotetraose is hydrolysed at ~10% the rate for sucrose.

An alternative approach (Quemener *et al.*, 1993) involves the use of capillary gas chromatography (CGC) or HPLC to analyse extracts of samples either non-treated, or treated with amyloglucosidase or amyloglucosidase plus inulinase (fructanase). By measuring sucrose, fructose and glucose in the various samples, and with appropiate calculations, it is possible to

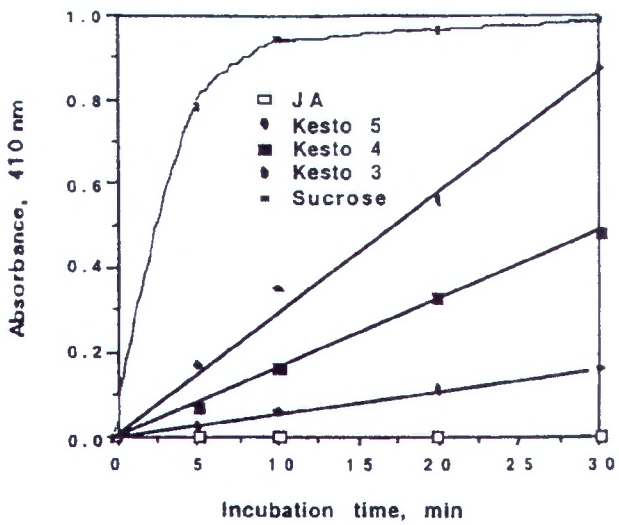

Fig. 1. Hydrolysis of sucrose and fructans by yeast invertase.
Sugar compound (0.2 ml, 50 µg) was incubated with invertase (2 units) in sodium acetate buffer (100 mM, pH 4.5) at 40 °C. Reaction was terminated at various time intervals with PAHBAH working reagent and colour developed. JA = Jerusalem artichoke inulin; Kesto 3 = 1-kestose; Kesto 4 = 1,1-kestotetraose; Kesto 5 = 1,1,1-kestopentaose.

determine free glucose and fructose, sucrose, starch and fructan. The possible interference of galactosyl-sucrose oligosaccharides (*e.g.* raffinose, stachyose and verbascose) which may be present in some mixed-food samples, was not considered. These oligosaccharides are hydrolysed by exo-inulinase to yield fructose and galactosyl-glucose oligosaccharides (*e.g.* melibiose from raffinose). Also, crude Fructozyme® (formerly known as Novozym SP 230) contains a very active α-galactosidase (see Table 1) which will rapidly hydrolyse galactosyl residues from any galactosyl-sucrose oligosaccharides present in the sample. This α-galactosidase is used in a commercially available test kit for the measurement of raffinose, stachyose and verbascose (Megazyme International). It would seem that the only way to avoid the possible interference of galactosyl-sucrose oligosaccharides in fructan assays, is to remove

Table 1. Levels of exo- and endo-inulinase, polygalacturonase (pectinase), cellulase and α-galactosidase in Fructozyme, heat-treated Fructozyme and a purified inulinase mixture (ex. Megazyme).

Enzyme	Enzyme activity (units ml^{-1})		
	Fructozyme	HT Fructozyme[a]	pure inulinase mixture
Exo-inulinase	2000	1200	2000
Endo-inulinase	200	200	200
Pectinase	40	4.1	0.4
Cellulase	4.0	4.0	0.1
α-Galactosidase	284	10.2	0.05

[a] The Fructozyme was pre-heated (HT Fructozyme) at 60 °C for 120 min. One unit of activity is the amount of enzyme required to release one micromole of product per min under standard assay conditions (40 °C, pH 4.5).

Table 2. Effect of addition of crude and purified iulinases on the recovery of inulin (fructan) in the AOAC International total dietary fibre procedure.

Enzyme preparation[a]	Quantity added	Recovery (%)	
		inulin (fructan)	β-glucan
Heat-treated Fructozyme	0.2 ml	0.2	11.4
	0.1 ml	0.2	52.0
	0.05 ml	7.2	80.0
Pure inulinase mixture	0.2 ml	0.2	99.0
	0.1 ml	0.2	101.0
	0.05 ml	3.5	98.5

[a] The levels of enzyme activities in the enzyme preparations are as shown in Table 1. In each case, the amount of inulin and β-glucan used in assays was 100 mg.

them by treatment with α-galactosidase in the initial incubation step (*i.e.* along with starch and sucrose). For most samples this treatment will not be necessary.

Separate from the possible problems with galactosyl-sucrose oligosaccharides, the method of Quemener *et al.* (1993) is quite complex, and requires the use of expensive equipment. Furthermore, it has been proposed (Quemener *et al.*, 1997) that this method can be integrated with the AOAC total dietary fibre method, by removing all insoluble fructan by treatment with heat-treated Fructozyme. This heat treatment did, as the authors claim, remove most of the pectinase enzyme activity, but it did not alter the level of cellulase contamination (Table 1) when assayed with Beta-Glucazyme tablets (containing dyed and cross-linked barley β-glucan; Megazyme International). This level of cellulase results in depolymerization, and thus underestimation, of β-glucan in samples in which this polymer is present. Recovery of β-glucan using the AOAC international total dietary fibre procedure (with high-purity amyloglucosidase, protease and thermostable α-amylase; Megazyme International) is shown in Table 2. Also shown is the recovery of β-glucan and fructan when heat-treated Fructozyme (*cf.* Quemener *et al.*, 1997) or a purified exo-inulinase/endo-inulinase preparation (with the same concentrations of these two enzymes as in the original Fructozyme preparation) is used. It is evident that the level of inulinase used in both cases is sufficient to remove the fructan. With the purified inulinase mixture, the recovery of β-glucan is quantitative, whereas with the heat-treated Fructozyme the recovery of β-glucan is dramatically reduced.

In the current paper, we (Blakeney *et al.*, 1997; McCleary and Blakeney, 1999; McCleary *et al.*, 1999; see also Megazyme kit booklet OFR9/97, 1997) describe a non-instrumental method for the measurement of fructan. This method simplifies fructan analysis, is easy to perform, uses standard laboratory equipment, and is accurate, reproducible and specific. This procedure employs highly purified and specific enzymes to hydrolyse sucrose, starch and fructans (inulins and graminan). The sucrase enzyme used in this method rapidly hydrolyses sucrose but has negligible activity on 1-kestose and other inulo-oligosaccharides (Fig. 2). Moreover, it has no action on inulobiose and inulotriose. At substrate concentrations of 10 mg ml^{-1}, the ratio of the relative rates of hydrolysis of sucrose and 1-kestose is 3,800 : 1.

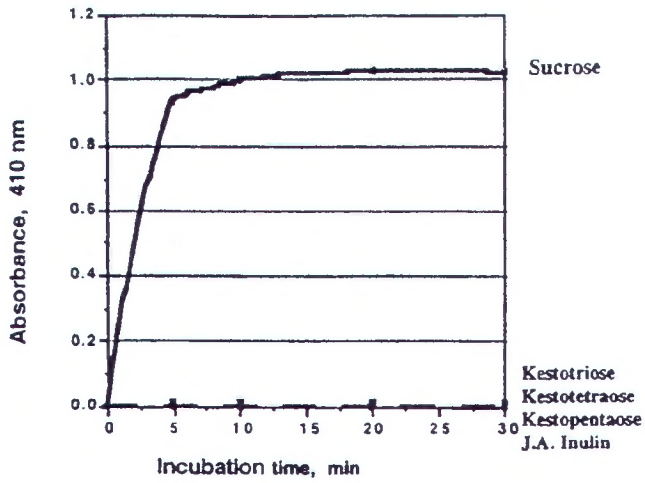

Fig. 2. Hydrolysis of sucrose and fructans by sucrase.
Sugar compound (0.2 ml, 50 µg) was incubated with sucrase (1 unit) in sodium maleate buffer (100 mM, pH 6.5) at 40 °C. Reaction was terminated at various time intervals with PAHBAH working reagent and colour developed.

2 PRINCIPLE OF THE FRUCTAN ASSAY PROCEDURE

The procedure for the measurement of fructan is described in detail in Megazyme kit booklet OFR9/97, and updated versions (see www.megazyme.com). The general principle of this procedure is outlined in Fig. 3. Sucrose is hydrolysed to glucose and fructose using a specific sucrase enzyme. Concurrently, starch and maltosaccharides (if present in the sample) are hydrolysed to glucose by the combined action of highly purified β-amylase (EC 3.2.1.2) (Megazyme cat. no. E-BARBL), pullulanase (EC 3.2.1.41) (Megazyme cat. no. E-PULKP) and yeast maltase (3.2.1.20) (Megazyme cat. no. E-MALTS) . These reducing sugars are then reduced to the sugar alcohols by treatment with alkaline borohydride. The solution is neutralized

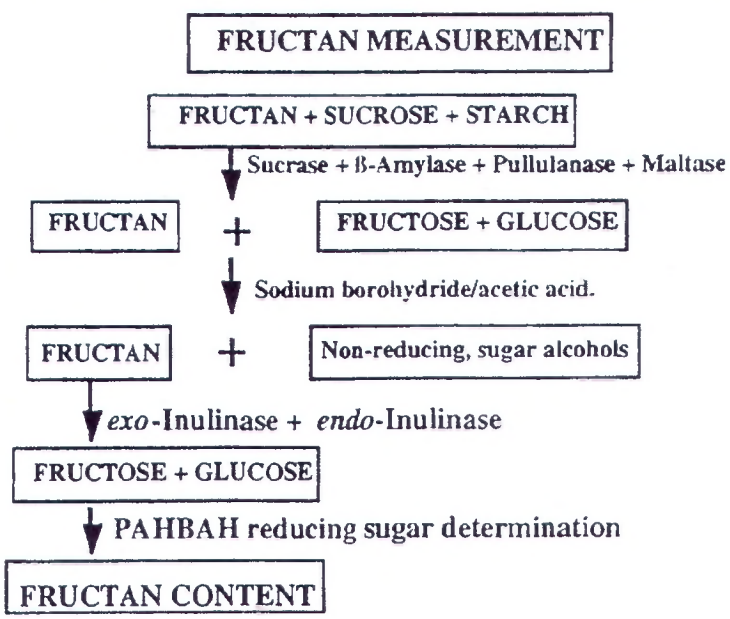

Fig. 3. Schematic outline of the current procedure for the measurement of inulin in plant and food products.

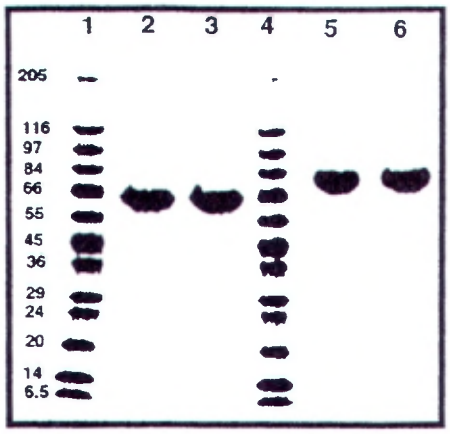

Fig. 4. SDS-gel electrophoresis of purified endo-inulinase and exo-inulinase.
Samples: 1 and 4, molecular weight standards; 2 and 3, endo-inulinase; 5 and 6, exo-inulinase.

and excess borohydride is removed by treatment with dilute acetic acid. The fructan is hydrolysed to fructose and glucose with purified fructanase (originally, a pure exo-inulinase [EC 3.2.1.80] was employed, but this was changed to a mixture of exo-inulinase and endo-inulinase [EC 3.2.1.7] [10:1]) and the reducing sugars produced are measured with the PAHBAH (p-hydroxy benzoic acid hydrazide) reducing-sugar method.

The exo- and endo-inulinase enzymes used in this work were both purified from Fructozyme by a combination of chromatographic procedures. Each enzyme appeared as a single band on SDS-gel electrophoresis, with molecular weights of 78,000 (exo-inulinase) and 65,000 (endo-inulinase), respectively (Fig. 4). On isoelectric focusing, the exo-inulinase appeared as several bands with pI values of 4.6-5.1. The endo-inulinase also appeared as several bands, but these were well separated from the exo-inulinase bands, with pI values of 3.5-3.7.

The difference in action patterns of these enzymes was clearly demonstrated by characterization of the reaction products on incubation with inulin or graminan. The time course of hydrolysis of three different fructans by exo-inulinase, together with the percentage hydrolysis values, is shown in Fig. 5. From the observation that the hydrolysate consists totally of partially hydrolysed polymer and fructose it is evident that this enzyme is an exo-enzyme. The enzyme brings about quantitative hydrolysis of each of the three fructans to fructose and glucose. In contrast, the action of endo-inulinase on high-molecular-weight fructan releases a series of oligosaccharides, with an intermediate accumulation of oligomers of DP (degree of polymerization) 4-7 (Fig. 6). On greatly extended incubation, these oligosaccharides are hydrolysed to glucose and fructose.

The fructan analytical method developed in the current research, as summarized in Fig. 3, is simple to use. The PAHBAH colour response with fructose and glucose is the same. Furthermore, with essentially the same format, it is possible to measure galactosyl-sucrose oligosaccharides. For these analyses, an extract of the sample (after borohydride reduction and neutralization) is treated with *Aspergillus niger* α-galactosidase and sucrase and the released reducing sugars are measured with the PAHBAH reducing-sugar method.

Glucose in the original extract can be measured directly using glucose oxidase/peroxidase reagent, whereas sucrose can be measured in the same way following treatment with sucrase.

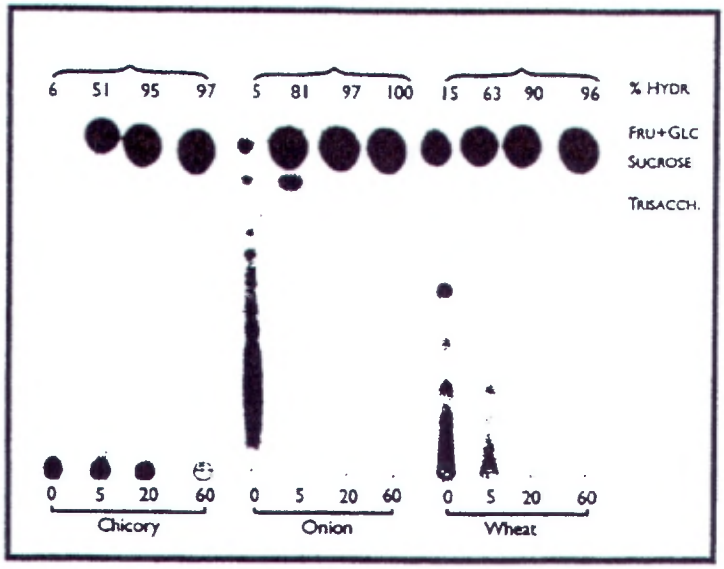

Fig. 5. Thin-layer chromatography of the products produced on hydrolysis of chicory, onion and wheat fructans by pure exo-inulinase.
The percentage hydrolysis values are the ratio of the reducing sugar and total carbohydrate values, given as a percentage. Fructan (5 g per 100 ml) in 10 mM sodium acetate buffer, pH 4.5, was incubated with 4000 units of exo-inulinase at 40 °C. Aliquots were removed at various time intervals and heated to 100 °C to inactivate the enzyme. Samples of this were analysed by TLC and sugar analysis methods. TLC plates were developed once with n-propanol-ethanol-water (7:1:2).

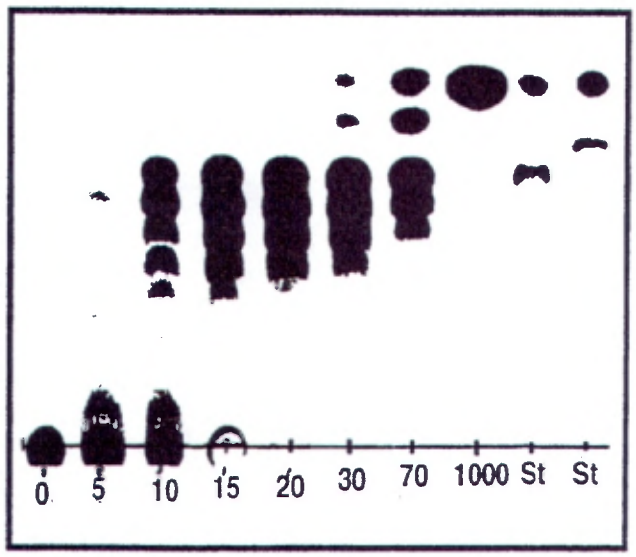

Fig. 6. Thin-layer chromatography of the oligosaccharides produced on hydrolysis of chicory inulin with pure endo-inulinase.
Chicory inulin (1 g per 50 ml) in 10 mM sodium acetate buffer, pH 5.0, was incubated with 50 units of endo-inulinase at 40 °C. Aliquots were removed at various time intervals and inactivated by heating to 100 °C. Samples were taken for TLC using the solvent described in Fig. 5. St1 (left) = sucrose + kestotetraose; St2 (right) = fructose and kestotriose.

5 APPLICATION OF THE FRUCTAN ASSAY PROCEDURE

A range of fructan-containing plant samples and food products to which fructan had been added, were analysed in duplicate on four separate days, and the results are shown in Table 3. All values are reported on an 'as is' basis.

This method has been the subject of an AOAC interlaboratory evaluation (McCleary *et al.*, 1999) and has been recommended for First Action Adoption (Anonymous, 1998).

6 SPECIFIC MEASUREMENT OF INULIN-DEGRADING ENZYMES

Enzymes involved in the depolymerization of inulin and graminan are exo-inulinases and endo-inulinases (Yun *et al.*, 1997). In general, inulinases have been assayed with inulo-oligosaccharide mixtures or a high-DP fraction of inulin (DP ~20-60). However, these substrates do not distinguish the types of activities involved. In the current study, as previously described, we have purified exo- and endo-inulinases from the commercial *Aspergillus niger* enzyme preparation Fructozyme to electrophoretic homogeneity, using a combination of anion and cation exchange chromatography and gel filtration (Yun *et al.*, 1997; McCleary and Blakeney, 1999).

6.1 Assay of exo-inulinase

Several substrates were evaluated for the assay of exo-inulinase, including dahlia inulin (high-DP fraction), kestotetraose, kestose and sucrose. It was assumed that an oligosaccharide such as kestose would not be hydrolysed by an endo-hydrolase, as these enzymes generally require a substrate of at least 3-4 homologous sugar residues. The relative initial rates of hydrolysis of sucrose, kestose, kestotetraose and dahlia fructan (each at 10 mg ml^{-1}) by exo-inulinase were 100, 100, 100 and 20.5, respectively. Rates were measured by increase in reducing-sugar levels using the Nelson-Somogyi method, and under incubation conditions where less than 10% of the initial substrate had been hydrolysed. Reducing values for sucrose were divided by 2 to allow for the fact that for each micromole of bonds broken, two micromoles of reducing sugars are released.

6.2 Assay of endo-inulinase

The relative rates of hydrolysis of sucrose, kestose and dahlia fructan by endo-inulinase were determined using these sugars each at 10 mg ml^{-1} in 0.1 M sodium acetate buffer, pH 4.5, as substrates, and employing the Nelson-Somogyi reducing-sugar procedure. Relative rates for sucrose, kestose and dahlia fructan were 14, 15 and 100, respectively. The high rates of hydrolysis of kestose and particularly of sucrose were totally unexpected. Obviously, the action pattern of this endo-acting enzyme is quite different to those of a whole range of other endo-acting enzymes (*e.g.* α-amylase, β-mannanase, β-glucanase, etc.) (McCleary and Matheson, 1987). This indicates that the enzyme might be contaminated with exo-inulinase. This seems unlikely, as the enzymes chromatographed quite differently on ion-exchange resins and on gel filtration. Also, their gel electrophoresis (Fig. 4) and isoelectric focusing patterns do not indicate cross-contamination.

This high action of endo-inulinase on kestose means that kestose cannot be used as a specific substrate for the assay of exo-inulinase. However, the significant relative rate ratios of hydrolysis of fructan compared to kestose for the two enzymes indicates that kestose is at least a

Table 3. Repeatability and reproducibility of the current fructan assay procedure with a range of samples.

Sample	Pure inulin (chicory)	Inulin/ cellulose[a]	Onion	Jerusalem artichoke	Vitamin tables	Butter spread	Chocolate	Milk powder	Wheat stems
Mean (4 days)	90.1	28.6	51.6	52.7	4.7	6.5	12.2	11.0	10.6
Standard deviation									
(rep)	0.84	0.15	0.69	0.63	0.24	0.17	0.89	0.27	0.45
(day)	1.58	1.65	1.77	2.08	0.25	0.61	0.81	0.24	0.38
(4 days)	1.47	1.65	1.70	2.03	0.17	0.59	0.50	0.14	0.21
Relative standard deviation									
(rep)	0.9	0.5	1.3	1.2	5.2	2.7	7.3	2.5	4.3
(day)	1.8	5.8	3.4	5.2	5.2	9.4	6.6	2.2	3.6
(4 days)	1.6	5.8	3.3	3.9	3.7	9.2	4.1	1.3	2.0

Samples were analysed on an 'as is' basis, *i.e.* there was no allowance for moisture content. The moisture content of these samples were: pure inulin, 9%; onion powder, 7.5%; Jerusalem artichoke, 5%; vitamin tablets, 2%; milk powder, 7.5%; and wheat stems, 8.2%. Butter spread and chocolate were not analysed for moisture content.

[a] The inulin/cellulose control was prepared by suspending α-cellulose in a solution of high-molecular-weight chicory inulin. The slurry was freeze-dried and milled.

good selective substrate for exo-inulinase.

Endo-enzymes can be specifically assayed in the presence of exo-acting enzymes using a range of assay procedures and modified substrates. Since polysaccharides usually give solutions of high viscosity, endo-enzymes can usually be easily distinguished from exo-enzymes by viscometric assays. However, fructans are low-DP 'polysaccharides', so they do not give solutions of high viscosity (at low polymer concentration). Consequently, viscometric assays would not be ideal.

Dye-labelled substrates are used routinely in the specific assay of polysaccharide endo-hydrolases, so we decided to evaluate the use of these. A dyed inulin was prepared by dyeing a high-DP inulin preparation (Raftiline HP, Orafti) with the azo-dye Remazol Brilliant Black. This is commercially available from Megazyme as 'Azo-Inulin'.

6.3 Assay of endo-inulinase using Azo-Inulin

Principle: The assay procedure is specific for endo-inulinase in the presence of exo-inulinase. The substrate is the high-molecular-weight fraction of chicory inulin dyed with the azo-dye. Exo-inulinase hydrolyses this substrate in an exo-fashion, and cannot hydrolyse the glycosidic linkage of a fructose molecule containing a dye molecule. However, because the fructan molecules are quite small (20-60 sugar units), the release of fructose from the non-reducing end of some molecules renders the remainder of these oligomers soluble in the presence of the precipitant solution. Thus, blank absorbance values in the absence of exo-inulinase are about 0.05 absorbance units, and in the presence of excess quantities of exo-inulinase (*e.g.* 20 units per assay) the blank absorbance value increases to about 0.23 absorbance units. In the standard assay procedure, Azo-Inulin solution is incubated (in the presence of excess exo-inulinase) with preparations containing endo-inulinase under defined assay conditions. The reaction is terminated and unhydrolysed Azo-Inulin is precipitated by the addition of an alkaline ethanolic

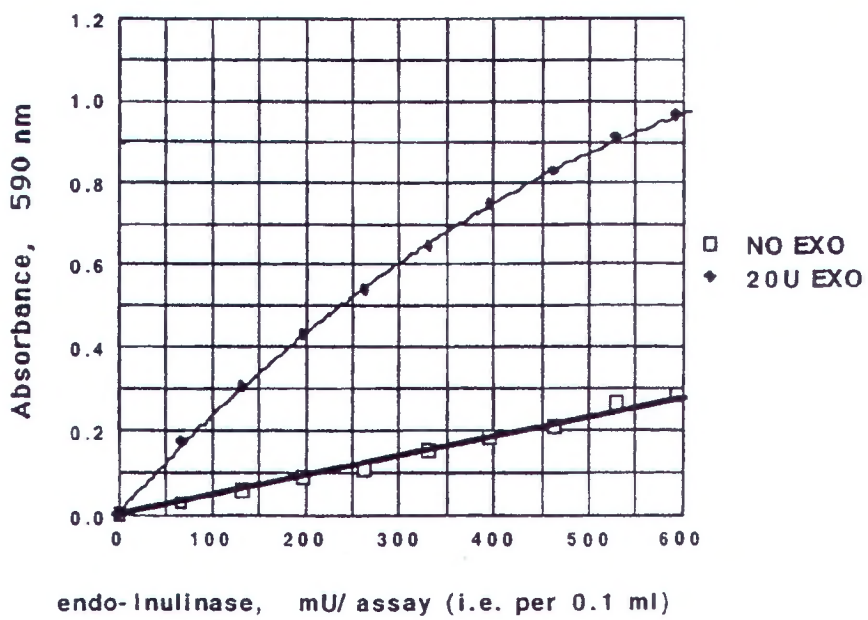

Fig. 7. Standard curve for endo-inulinase on Azo-Inulin (lot 80201).
Assays were performed in the presence and absence of excess quantities (20 units) of exo-inulinase. One unit of activity is defined as the amount of enzyme required to release one micromole of reducing-sugar equivalents per min under defined assay conditions.

solution. The solution is stirred vigorously, centrifuged and the absorbance of the supernatant solution measured at 590 nm. The activity of endo-inulinase is determined by reference to a standard curve (Fig. 7). It is evident that the presence of exo-inulinase affects the sensitivity of the assay. Basically, as the endo-inulinase hydrolyses the fructan molecule, it exposes regions of fructan which are susceptible to exo-inulinase. On action of exo-inulinase, smaller dyed fragments are produced and these become soluble in the precipitant solution.
Standard curve: A typical standard curve is shown in Fig. 7. This curve is for pure *Aspergillus niger* endo-inulinase diluted in 0.1 M sodium acetate buffer (pH 4.5) on Azo-Inulin lot 80201. Enzyme activity is standardized using dahlia fructan (10 mg ml^{-1}) as substrate in 100 mM sodium acetate buffer (pH 4.5) at 40 °C using the Nelson-Somogyi reducing-sugar method.

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