Appl Biochem Biotechnol (2009) 158:352–361
DOI 10.1007/s12010-009-8596-x

Hydrolytic Methods for the Quantification of Fructose Equivalents in Herbaceous Biomass

Stefanie K. Nguyen · Supaporn Sophonputtanaphoca · Eugene Kim · Michael H. Penner

Received: 23 May 2008 / Accepted: 26 February 2009 /
Published online: 31 March 2009

Abstract A low, but significant, fraction of the carbohydrate portion of herbaceous biomass may be composed of fructose/fructosyl-containing components ("fructose equivalents"); such carbohydrates include sucrose, fructooligosaccharides, and fructans. Standard methods used for the quantification of structural-carbohydrate-derived neutral monosaccharide equivalents in biomass are not particularly well suited for the quantification of fructose equivalents due to the inherent instability of fructose in conditions commonly used for hemicellulose/cellulose hydrolysis (>80% degradation of fructose standards treated at 4% sulfuric acid, 121°C, 1 h). Alternative time, temperature, and acid concentration combinations for fructan hydrolysis were considered using model fructans (inulin, β-2,1, and levan, β-2,6) and a grass seed straw (tall fescue, *Festuca arundinacea*) as representative feedstocks. The instability of fructose, relative to glucose and xylose, at higher acid/temperature combinations is demonstrated, all rates of fructose degradation being acid and temperature dependent. Fructans are shown to be completely hydrolyzed at acid concentrations well below that used for the structural carbohydrates, as low as 0.2%, at 121°C for 1 h. Lower temperatures are also shown to be effective, with corresponding adjustments in acid concentration and time. Thus, fructans can be effectively hydrolyzed under conditions where fructose degradation is maintained below 10%. Hydrolysis of the β-2,1 fructans at temperatures ≥50°C, at all conditions consistent with complete hydrolysis, appears to generate difructose dianhydrides. These same compounds were not detected upon hydrolysis of levan, sucrose, or straw components. It is suggested that fructan hydrolysis conditions be chosen such that hydrolysis goes to completion; fructose degradation is minimized, and difructose dianhydride production is accounted for.

Keywords Fructose · Fructans · Fructooligosaccharides · Inulin · Levan · Lignocellulosics · Hydrolysis

Introduction

There is widespread interest in expanding the use of lignocellulosic biomass as a means of enhancing global bio-based economies. This interest has fostered research programs

S. K. Nguyen · S. Sophonputtanaphoca · E. Kim · M. H. Penner (✉)
Food Science and Technology, Oregon State University, 100 Wiegand Hall, Corvallis, OR 97331-6602, USA
e-mail: mike.penner@oregonstate.edu

directed at the development of processes for the production of biofuels from a broad spectrum of lignocellulosic starting materials [1]. A necessary component of essentially all such research programs is an ability to monitor the changes in the major chemical components of the starting materials. In many cases, the components of primary interest are the carbohydrates, which are present in large amounts and may be processed to generate relatively simple sugars for subsequent fermentation and/or chemical transformation.

In this study, we have focused on the quantitative determination of the fructose equivalents in lignocellulosic materials. "Fructose equivalents," in the context of this study, refers to the amount of fructose theoretically available for subsequent processing following complete hydrolysis of the fructosyl-containing polysaccharides to their constituent monosaccharides. The analytical approach considered herein is the same as that used to quantify the analogous glucose or xylose equivalents, hydrolysis of the parent polysaccharides followed by chromatographic separation of the resulting monosaccharides and quantification using an appropriate detector. The amount of fructose equivalents in lignocellulosic materials is typically not high relative to glucose and xylose, but it can be significant. Chen et al. [2] recently showed monomeric sugars (primarily glucose and fructose) of corn stover accounting for 30–46% of the dry weight extractives (4–12% of the dry weight of feedstocks) and herein we show that fructose equivalents represent a significant fraction of a representative grass seed straw. Carbohydrate equivalents in lignocellulosic biomass are most commonly quantified using the two-stage hydrolysis method developed for the quantification of structural carbohydrates [3]. This procedure entails an initial 72% sulfuric acid treatment followed by a 4% sulfuric acid, 121°C, treatment for 1 h. The drawback of using this method for the determination of fructose equivalents is that fructose is relatively unstable, compared to other monosaccharides typically analyzed, under these hydrolysis conditions. Thus, the correction factors used in calculating the number of fructose equivalents in a feedstock, determined by measuring the extent of degradation of a known amount of pure fructose under equivalent hydrolysis conditions, are excessive. It is generally recognized that these correction factors should be kept to a minimum since they provide only approximations of actual sugar losses [4].

In the present study, we have evaluated time, temperature, and acid concentration relationships that are pertinent to the hydrolysis and subsequent quantification of fructose equivalents in lignocellulosic biomass. The study considered temperatures to 121°C and sulfuric acid concentrations to 4%; these being the conditions specified for the secondary hydrolysis step of traditional methods [3]. Conditions were thus considered in the context of established hydrolysis parameters that are used for the quantification of neutral sugar equivalents in lignocellulosic biomass. The hypothetical basis of this study is that hydrolyses using relatively low acid concentrations and temperatures are preferable for the quantification of fructose equivalents due to the relative ease of hydrolysis of fructosidic linkages and the relative instability of fructose under low-pH/high-temperature conditions.

Materials and Methods

Materials

Sugar standards: inulin, levan, sucrose, glucose, and fructose were obtained from Sigma (USA). Levan was also purchased from Montana Polysaccharides Corporation (USA). Tall fescue grass seed straw was obtained from commercial Pacific Northwest grass seed farms. Straws had 6–8% moisture when taken from the field. Straws were milled to pass a 20-mesh-per-inch screen. The milled and sieved straw was stored under dry conditions at room temperature prior to use.

Monosaccharide Decomposition Studies

Sugars were dried at 45°C for 12 h and stored with desiccant prior to their use. Sugar solutions containing 1 mg/mL of the test sugars at specified acid concentrations were prepared just prior to testing. Acid concentrations ranged from 0.02% to 4.00% (wt/wt, 0.002 to 0.418 M). Molar concentrations of the acid solutions were determined by titration with standardized base (0.1 N NaOH) to the phenolphthalein end point.

Autoclave and Water Bath Hydrolysis Method

For the autoclave hydrolysis method, sugar solutions (25 mL of 1 mg/mL in appropriate acid concentration) were transferred to 100-mL autoclavable, Teflon-lined, screw-capped bottles. The samples were then autoclaved at 121°C for 1 h—the 1-h time period started once the autoclave had reached 121°C. For the water bath hydrolysis method, equivalent sugar solutions were placed in an appropriate temperature-regulated water bath and allowed to react for the given time periods—the timed periods started once the bottles were immersed in the water bath. Following the hydrolysis period, the samples were transferred to an ice bath for rapid cooling. Once the samples approximated room temperature, they were transferred to 50-mL flasks and solid $CaCO_3$ was added to raise the pH to between 6 and 7 (determined by pH meter). Samples were then set aside for 60 min, at room temperature, to allow precipitate development and the settling of solids. All experiments were done in triplicate.

Hydrolysis of Inulin and Levan

Inulin and levan solutions containing 1 mg/mL of inulin or levan at specified acid concentrations were prepared and hydrolyzed as described above. All experiments were done in triplicate.

Tall Fescue Extraction and Hydrolysis

Approximately 6 g of sample, weighed to the nearest 0.1 mg, were placed in a 250-mL screw-top Erlenmeyer flask containing 94 mL of double-distilled pre-heated water. The flask was maintained at 60°C for 2 h in thermoregulated water bath with orbital mixing at 200 rpm. At the completion of the extraction, the contents were cooled and filtered using a Whatman filter paper no. 1. Approximately 15–20 g of filtrate, weighed nearest to 0.1 mg, was transferred to a pre-dried weighed porcelain crucible and dried in a convection oven at 105°C for 12 h for "solids" determination. The filtered tall fescue extract was hydrolyzed using the autoclave and water bath methods as described above. Hydrolysis was done on 10 mL of filtrate that was diluted to 25 mL with aqueous sulfuric acid to give the appropriate acid concentration. All experiments were done in triplicate.

HPLC

Prior to high-performance liquid chromatography (HPLC) analyses, portions of the sample hydrolyzates were filtered through 0.2-μm Acrodisc© syringe filters (Pall, USA) into auto-sampler vials. Analyses were done using a Waters HPLC system equipped with Aminex HPX-87P column (300×7.8 mm, Bio-Rad, USA) and a refractive index detector. Analysis conditions were as follows: injection volume, 20 μL; mobile phase, Milli-Q grade H_2O; flow rate, 0.6 mL/min; column temperature, 85°C; running time, 50 min.

Levan Purification

Crude levan (from Montana Polysaccharide Corp.), 5 g, was washed three times by suspending in 25 mL of 100% ethanol for 15 min. The washed levan was then dissolved in 50 mL of deionized water, stirred for 20 min, filtered (Whatman no. 1 filter paper), and then re-precipitated by the addition of 500 mL 100% ethanol. The suspension was centrifuged for 10 min at 10,000 rpm and the supernatant was decanted and discarded. This process was repeated a second time. The sample was then washed with absolute ethanol and dried for 16 h in a convection oven at 37°C. The dried levan was stored in a desiccator until ready for use.

Results and Discussion

Figure 1 illustrates the relative lability of fructose at high-temperature/low-pH conditions (121°C, acid concentrations to 4%) and, thus, the motive for this study. Treatment of fructose solutions using traditional secondary hydrolysis conditions, 4% acid/121°C/1 h, results in the degradation of nearly 90% of the initial fructose. For reference, the degradation of glucose and xylose under these same conditions is typically less than 10% and 20%, respectively. Hence, the correction factor used in calculating the amount of the monosaccharide theoretically available in a feedstock, using these hydrolysis conditions, is inordinately large for fructose (for discussion of use of correction factors based on degradation of standard monosaccharide solutions, see [3]). Figure 2 illustrates that there is significant loss of fructose from aqueous solutions at 121°C even in the absence of acid. These results prompted our consideration of hydrolysis at lower temperatures; representative data from such experiments at 100°C are included in Fig. 1. The data show that the extent of fructose degradation was 10% or less when solutions were incubated at 100°C for 1 h at acid concentrations up to 1% (the stability at higher acid concentrations was not tested because 1% acid was sufficient for hydrolysis of the tested fructan linkages, as discussed below). Yet lower temperatures were tested (50°C for 1 h at acid concentrations up to 4%); under these conditions, fructose degradation was not observed, neither by a significant decrease in measured fructose nor the generation of hydroxymethylfurfural (HMF).

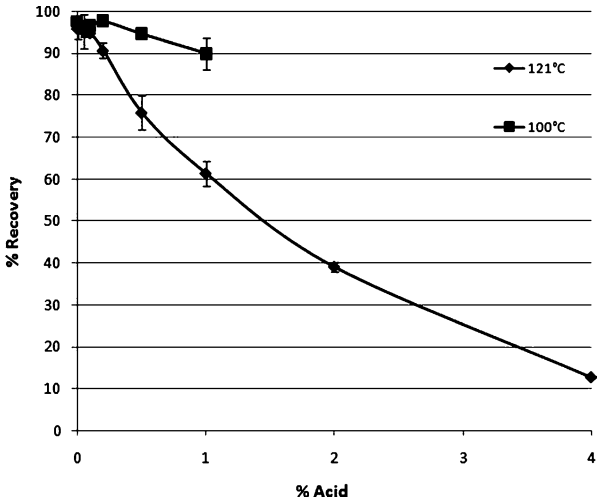

Fig. 1 Fructose degradation after 1 h at different acid and temperature combinations. *Error bars* ±1 standard deviation

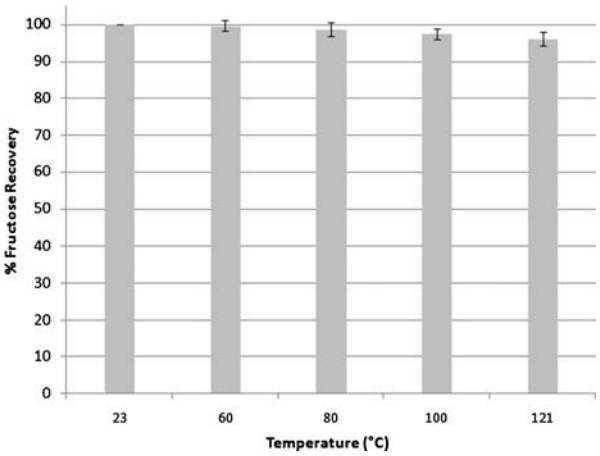

Fig. 2 Fructose degradation in water after 1 h at different temperatures. *Error bars* ±1 standard deviation

The primary linkages in fructans from grass and cereal straws are expected to be β-2,1 and β-2,6 [5]. Hence, our hydrolysis studies used inulin as a model β-2,1 fructan and levan as a model β-2,6 fructan. Initial studies with inulin, summarized in Figs. 3, 4, 5, 6, 7, and 8, demonstrate that it is readily hydrolyzed, compared to the structural polysaccharides, under rather modest conditions. At 121°C for 1 h, inulin was effectively hydrolyzed at acid concentrations down to 0.1% (Fig. 3). Shorter reaction times, at 121°C, were not considered due to the common use of the 1-h treatment for the hydrolysis of structural polysaccharides (typically 4% acid/121°C/1 h). Time and acid combinations at 100°C showed that the β-2,1 fructan was hydrolyzed in as short as 10 min at 0.5% acid (Fig. 4) or at acid concentrations as low as 0.1% if treated for 1 h (Fig. 5). Experiments done at yet lower temperatures, 50°C, with incubation times of 1 h and acid concentrations of 0.2%, 0.5%, and 1.0% resulted in extents of inulin hydrolysis of 33%, 72%, and 96%, respectively (Fig. 6); treatments at 50°C for 1 h at the higher acid concentrations (≥2%) resulted in complete hydrolysis (Fig. 6). Hydrolysis at the lower acid concentrations, at 50°C, also revealed sucrose as an intermediate hydrolysis product, as expected based on the ease of hydrolysis of inulobiose relative to sucrose [6], there being one potential sucrose moiety per molecule inulin. The apparent decrease in overall accountable mass under the mildest hydrolysis conditions depicted in Figs. 6 and 7 suggests that, under these mildest conditions, a range of

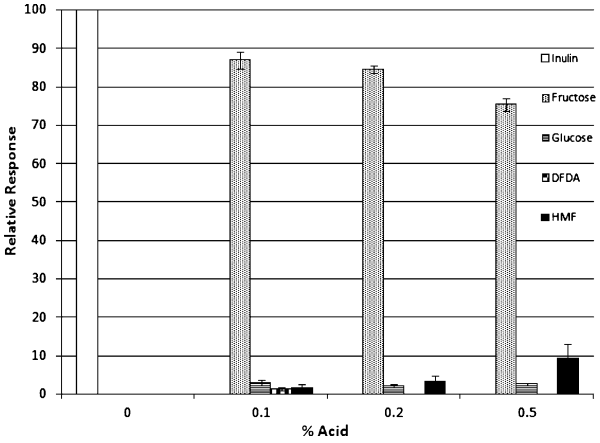

Fig. 3 Time course of inulin hydrolysis at 121°C, 1 h, at different concentrations of sulfuric acid. *DFDA*, difructose dianhydride; *HMF*, hydroxymethylfurfural. "Relative response" is relative to 1 mg/mL fructose or glucose solution. *Error bars* ±1 standard deviation

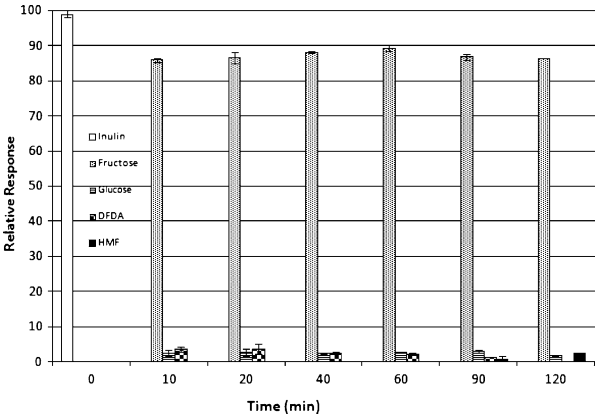

Fig. 4 Time course of inulin hydrolysis at 100°C, 0.5% sulfuric acid. *DFDA*, difructose dianhydride; *HMF*, hydroxymethylfurfural. "Relative response" is relative to 1 mg/mL fructose or glucose solution. *Error bars* ±1 standard deviation

intermediate-molecular-weight inulooligosaccharides are present at levels below the detection limit of the system used in this study.

The data of Figs. 3, 4, 5, 6, 7, and 8 illustrate a potential complication in using acid as the catalyst for the hydrolysis of inulin-type fructans, the complication being the production of a hydrolysis byproduct which we have tentatively identified as difructose dianhydrides (DFDA). The tentative identification is based on the observation that the compound is relatively resistant to acid-catalyzed hydrolysis; the only detectable product resulting from its hydrolysis is fructose; it is resistant to an enzyme preparation for fructan hydrolysis ("Fructozyme," Sigma Chemical Co.); its retention time is consistent with that of disaccharides, and DFDA are known to be generated as a result of acid-catalyzed hydrolysis of 2,1-linked fructans [7]. The presence of DFDA presents a complication in quantifying fructose equivalents for inulin-type fructans because they are not accounted for by traditional correction factors. The correction factors used when quantifying structural-carbohydrate-derived neutral sugars are based on the degradation of the monosaccharides of interest (under the chosen hydrolysis conditions). This approach will not work for the generation of DFDA since they are apparently not formed from the monosaccharide under the conditions used in this study but are derived directly from inulin (we see no evidence for the formation of DFDA when fructose alone is incubated at the hydrolysis conditions evaluated in this study).

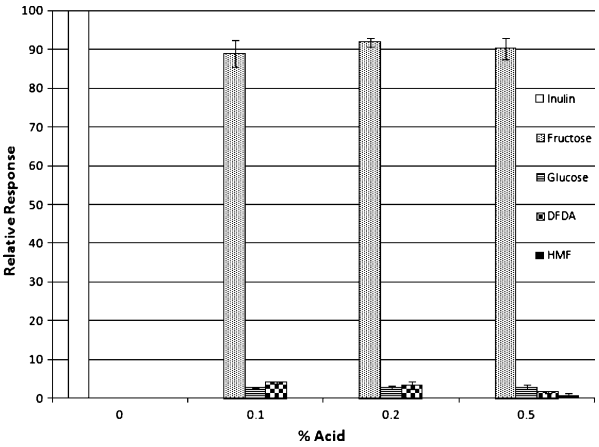

Fig. 5 Hydrolysis of inulin at 100°C, 1 h, at different concentrations of sulfuric acid. *DFDA*, difructose dianhydride; *HMF*, hydroxymethylfurfural. "Relative response" is relative to 1 mg/mL fructose or glucose solution. *Error bars* ±1 standard deviation

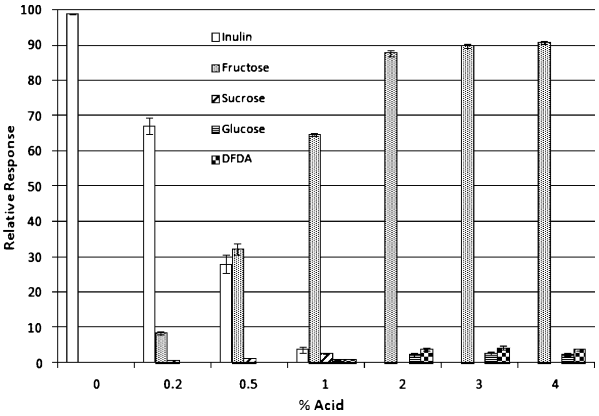

Fig. 6 Hydrolysis of inulin at 50°C, 1 h, at different concentrations of sulfuric acid. *DFDA*, difructose dianhydride. "Relative response" is relative to 1 mg/mL fructose or glucose solution. *Error bars* ±1 standard deviation

Many time–temperature–acid concentration combinations were tested in an attempt to avoid DFDA production. In general, the lower-temperature treatments appeared most promising. However, the data of Fig. 7, which summarize the time course of hydrolysis of inulin at a relatively low temperature, 50°C, 0.5% H_2SO_4, suggest that DFDA are generated prior to the completion of inulin hydrolysis. Experiments at yet a lower temperature 20°C, 5% acid, showed no sign of DFDA production after 4 h of incubation (data not shown)—but the calculated pseudo half-life for total inulin hydrolysis under these conditions was greater than 4 h. Therefore, these conditions were deemed unreasonable for routine analyses based on the extensive time it would require for "complete" hydrolysis. DFDA production at 20°C, 5% acid, was thus not tested beyond 4 h. Note that DFDA production is estimated to be 5% or less under all conditions tested in this study.

DFDA are relatively resistant to hydrolysis but upon hydrolysis are expected to yield fructose [7, 8]. Thus, one approach to accounting for their presence is to use hydrolysis conditions sufficiently harsh to hydrolyze the DFDA (essentially treating the DFDA as an uncommon intermediate in the hydrolysis scheme). This approach is summarized in Fig. 8, which depicts the time course of inulin hydrolysis at 100°C and 1% acid. It can be noted that complete hydrolysis of the DFDA occurred after 90 min. Similarly, the data of Fig. 3 show that no DFDA was present following hydrolysis at 121°C, for 1 h, at acid

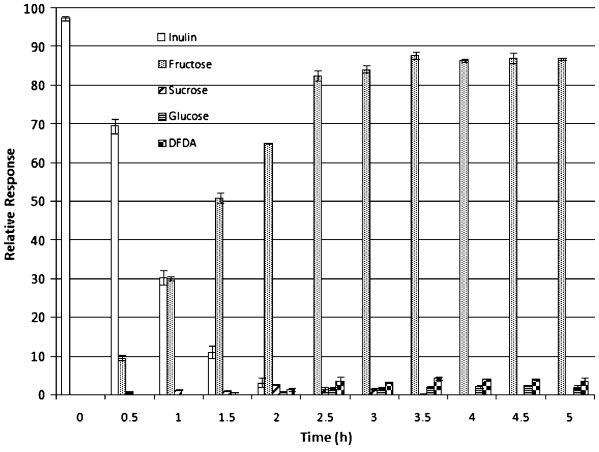

Fig. 7 Time course of inulin hydrolysis at 50°C, 0.5% sulfuric acid. *DFDA*, difructose dianhydride. "Relative response" is relative to 1 mg/mL fructose or glucose solution. *Error bars* ±standard deviation

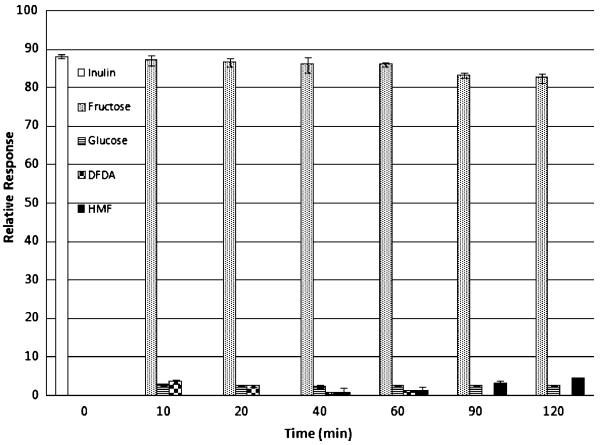

Fig. 8 Time course of inulin hydrolysis at 100°C, 1% sulfuric acid. *DFDA*, difructose dianhydride; *HMF*, hydroxymethylfurfural. "Relative response" is relative to 1 mg/mL fructose or glucose solution. *Error bars* ±1 standard deviation

concentrations of ≥0.2%. It is to be kept in mind that fructose degradation (principally to HMF) increases as the severity of the hydrolysis conditions increase. Fructose degradation of this nature, i.e., degradation via HMF, may be accounted for by using the traditional correction factors (standard fructose solutions incubated under equivalent conditions)—with the objective of keeping the extent of degradation as low as possible [4].

The levan-type fructans (2,6-fructans) were found to be readily hydrolyzed under the same conditions found effective for hydrolysis of the 2,1-type fructans. Thus, effectively all of the levan was hydrolyzed following treatment at 100°C with 0.1% acid for 1 h. The hydrolysis of levan produced no detectable DFDA, allowing the choice of conditions for hydrolysis of 2,6-fructans to be dictated simply by time of hydrolysis and extent of fructose degradation.

Sucrose, another potential source of fructose equivalents, can be a significant component of herbaceous feedstocks, as demonstrated by the data of Fig. 9 for an extract of tall fescue straw (discussed below) and as recently shown for corn stover [2]. Studies evaluating the hydrolysis of sucrose at relatively low acid concentrations, 0.2% acid, and 100°C demonstrate that it is effectively hydrolyzed at 10 min under these conditions (Fig. 10). The data contained in Fig. 9 demonstrate that the sucrose component of tall fescue is effectively hydrolyzed within 10 min in 1% acid at 100°C. Similarly, the inulin-derived

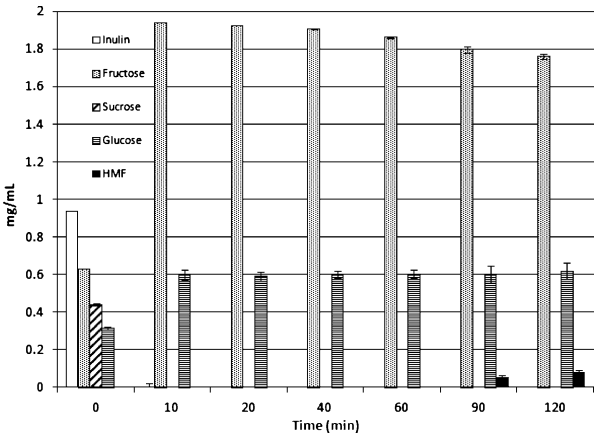

Fig. 9 Time course of hydrolysis of tall fescue extract at 100°C, 1% sulfuric acid. *HMF*, hydroxymethylfurfural. "Relative response" is relative to 1 mg/mL fructose or glucose solution. *Error bars* ±1 standard deviation

sucrose, noticeable during the time course of inulin hydrolysis at 50°C, 0.5% acid, was found to be completely hydrolyzed in 130 min (Fig. 7).

All of the data summarized above suggest that the major fructose-containing compounds are readily hydrolyzed in 1% acid at 100°C and that the rate of fructose degradation under these conditions is low, relative to rates of hydrolysis. The fructose equivalents of a representative grass seed straw (tall fescue) were thus determined using these conditions, with a 1-h incubation period. In this case, i.e., the analyses of fructose equivalents in tall fescue straw, there was no evidence for the production of DFDA during hydrolysis and, hence, the fructose equivalents could be determined simply by the application of the appropriate correction factor (~10% of the standard fructose solution was lost under these hydrolysis conditions). Using this approach, the analysis indicated that the straw contained ~76 g fructose equivalents per kilogram of straw (dry weight basis).

The above value for grams of fructose equivalents per kilogram of straw is based on analysis of the fructose equivalents in a 60°C water extract of the straw (see "Materials and Methods"). A separate analysis was done by directly hydrolyzing the straw, without fractionation by water extraction. The results from the two approaches were not significantly different; the value obtained by hydrolysis of a water extract of the straw was 76.5±1.3 and the value obtained by directly hydrolyzing the unextracted straw was 75.9±2.9. It is generally assumed that the fructans are water soluble; thus, it is common to see an extraction prior to hydrolysis [9]. If extractions are to be done, then it is important to again consider the temperature. As noted above, there was detectable degradation of fructose in water at 121°C after 1 h. Further experiments at 100°C showed a 10% decrease in fructose content of a standard fructose solution after 6 h at this temperature (data not shown). This suggests, where possible, extended extraction times at high temperatures are to be avoided due to the potential fructose degradation under such conditions. This caution is pertinent to the use of Soxhlet-type apparatus for extended periods.

The data summarized above provide information that may be used in deciding the appropriate conditions for hydrolysis when attempting to quantify fructose equivalents. It is generally recognized that degradation correction factors are to be kept to a minimum when determining monosaccharide equivalents [4]. This suggests that the hydrolysis conditions typically employed for hydrolysis of structural carbohydrates are not appropriate for the hydrolysis of fructans and sucrose. We have shown a number of milder conditions that may be employed for such hydrolyses. The analysis of the fescue straw was done using 1% acid,

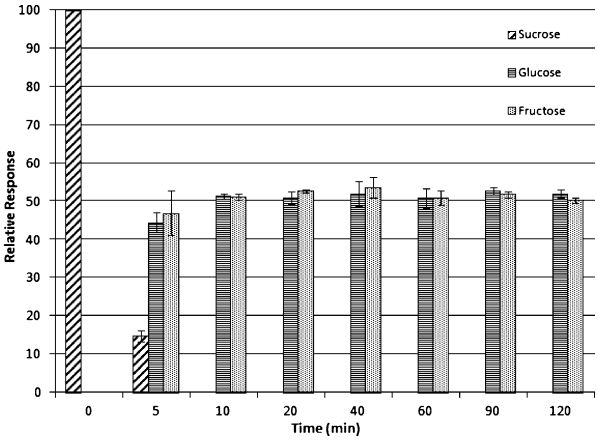

Fig. 10 Time course of sucrose hydrolysis at 100°C, 0.2% sulfuric acid. "Relative response" is relative to 1 mg/mL fructose or glucose solution. *Error bars* ±standard deviation

100°C, 1-h incubation, because this combination resulted in relatively low fructose degradation (<10%) and this acid/temperature combination is sufficient for the timely hydrolysis of DFDA should it be present in the hydrolyzate.

References

1. Huber, G. W., Iborra, S., & Corma, A. (2006). *Chemical Reviews, 106*, 4044–4098. doi:10.1021/cr068360d.
2. Chen, S. F., Mowery, R. A., Scarlata, C. J., & Chambliss, C. K. (2007). *Journal of Agricultural and Food Chemistry, 55*(15), 5912–5918. doi:10.1021/jf0700327.
3. National Renewable Energy Laboratory (NREL). (2008). Determination of structural carbohydrates and lignin in biomass, laboratory analytical procedure (LAP): Technical Report, NREL/TP-510-42618. NREL, Golden, CO. http://www.nrel.gov/biomass/analytical_procedures.html.
4. Puls, J. (1993). In J. N. Saddler (Ed.), *Bioconversion of forest and agricultural plant residues, vol. 9*, pp. 13–32. Wallingford: CAB International.
5. Bonnett, G. D., Sims, I. M., Simpson, R. J., & Cairns, A. J. (1997). *The New Phytologist, 136*(1), 11–17. doi:10.1046/j.1469-8137.1997.00721.x.
6. Heyraud, A., Rinaudo, M., & Taravel, F. R. (1983). In A. Strub, P. Chartier & G. Schleser (Eds.), *Energy from Biomass—2nd E.C. Conference*, pp. 883–888. London: Applied Science Publishers.
7. Kawamura, M., & Uchiyama, T. (2007). In S. Norio, N. Benkebelia & S. Onodera (Eds.), *Recent advances in fructooligosaccharides research*, pp. 273–296. Kerala: Research Signpost.
8. Manley-Harris, M. (1997). *Advances in Carbohydrate Chemistry and Biochemistry, 52*, 207–239. doi:10.1016/S0065-2318(08)60092-X.
9. Chatterton, N. J., & Harrison, P. A. (2003). *Journal of Plant Physiology, 160*, 843–849. doi:10.1078/0176-1617-01127.