Determination and Identification of Nonstructural Carbohydrates Removed from Grass and Legume Tissue by Various Sulfuric Acid Concentrations, Takadiastase, and Water

R. D. Groteluesche[1] and Dale Smith

In addition to glucose, fructose, and sucrose, water extracted the fructosans from timothy, and maltose, raffinose, and some dextrins from alfalfa. Only glucose was obtained when the residue from water extraction was hydrolyzed with takadiastase. Hot 0.005*N* H_2SO_4 completely hydrolyzed the fructosans and sucrose to fructose and glucose, and also fructose was obtained from raffinose and arabinose from hemicellulose. Further hydrolysis of hemicellulose and hydrolysis of starch was first detected with 0.2*N* H_2SO_4. Hydrolysis of starch was still incomplete with 0.8*N* H_2SO_4. Some destruction of fructose was apparent with 0.2 and 0.8*N* H_2SO_4. Enzymatic analysis offers the most specific and accurate method for determination of total nonstructural carbohydrates, especially with tissue high in starch.

An estimate of the content of nonstructural carbohydrates in the stem bases and roots of forage plants is often an integral part of cutting and grazing studies, especially as related to the total carbohydrates readily available for regrowth. The importance of carbohydrate reserves in management studies was recently reviewed by Sonneveld (1962).

Techniques to remove the total nonstructural carbohydrates from plant tissue have involved water (Baker and Garwood, 1961; Okajima and Smith, 1964; Waite and Boyd, 1953), varying concentrations of acids (Smith *et al.*, 1964), and various preparations of amylases (Graber *et al.*, 1927; Smith and Graber, 1948; Webster *et al.*, 1963; Weinmann, 1947). Water has proved to be a suitable extractant only when fructosans predominate, since starch is not readily soluble in cold water. Amylase preparations, such as takadiastase or saliva, have been used for the hydrolysis of starch (Smith and Graber, 1948; Weinmann, 1947). Webster *et al.* (1963) stated that takadiastase hydrolyzes much more than starch from grasses at certain stages of growth. Although these methods are widely used, few data are available as to the carbohydrate components removed. It is not known whether the nonstructural carbohydrates are completely removed from the tissue or whether some of the structural components—e.g., hemicelluloses—may also be removed.

The present study used thin-layer and paper chromatography to determine the carbohydrate fractions present following treatment of the tissue with water, acid, and takadiastase. Alfalfa (*Medicago sativa* L.) root and timothy (*Phleum pratense* L.) stem base tissues were used because starch (Graber *et al.*, 1927) and fructosans (Harper and Phillips, 1943; Okajima and Smith, 1964) are the predominant nonstructural carbohydrates stored by these species, respectively.

MATERIALS AND METHODS

A sample of timothy stem base tissue and another of alfalfa root tissue, both high in stored carbohydrates, were dried at 70° C. and ground to 40-mesh size. The nonstructural carbohydrates were removed from the tissue with cold water, a takadiastase enzyme preparation (Clarase 900, Miles Chemical Co., Elkhart, Ind.), and hot 0.005*N*, 0.02*N*, 0.2*N*, and 0.8*N* H_2SO_4 as described previously (Smith *et al.*, 1964). After filtering off the residue, the total nonstructural carbohydrate values for the samples hydrolyzed with the various acid concentrations were determined directly on the filtrates. Total nonstructural carbohydrate values for the samples extracted with water and those treated with takadiastase were obtained after filtering off the residue and hydrolyzing the filtrates with 0.1*N* H_2SO_4. Reducing sugars were determined by analyzing an aliquot of the water and takadiastase filtrates prior to acid hydrolysis. The residue remaining after cold water extraction was also treated with takadiastase. Total nonstructural carbohydrate and reducing sugar values were determined with the Shaffer-Somogyi copper-iodometric titration method (Heinze and Murneek, 1940). Fructose and glucose standard solutions were used for timothy and alfalfa, respectively.

Individual sugars in all of the filtrates were separated by thin-layer chromatography (TLC), except the filtrates obtained by direct takadiastase hydrolysis where the purification procedure would have hydrolyzed the sucrose and fructosans (Lewis, 1965). The acid filtrates were neutralized to pH 6.5 prior to concentration, separation, and analysis of the individual sugars (Smith and Groteluesche, 1966). The sugars in the acid filtrates were also analyzed after separation by paper chromatography (Moore *et al.*, 1960; Saeman *et al.*, 1954) to obtain galactose, mannose, and xylose values. Prior to paper chromatography, the acid filtrates were neutralized to pH 4.0 by adding concentrated $Ba(OH)_2$ with rapid stirring. The $BaSO_4$ was centrifuged from the solution and an aliquot of the supernatant was concentrated by lyophilization. Paper chromatography separated all sugars assayed except mannose, fructose, and arabinose. These three sugars were evaluated by analysis of the eluate from the paper chromatograms containing these sugars by three methods: (*a*) phloroglucinol reaction (630 mμ), utilizing 0.2% $FeCl_3$ in the primary reaction and reheating 4 minutes with phloroglucinol in the secondary reaction (Lindh, 1957), and (*b* and *c*) separate ferricyanide reductions (Furuholmen *et al.*, 1964) at 55° and 100° C.

Department of Agronomy, University of Wisconsin, Madison, Wis. 53706.

[1] Present address, Agronomy Department, Engineering Research, Deere and Co., Moline, Ill. 61265.

Specific carbohydrate concentrations in the acid filtrates were determined by proportionately adjusting the individual sugar concentrations with the actual concentration of glucose obtained by direct analysis of the filtrates with Glucostat special reagent, a coupled glucose-oxidase enzyme system (Worthington Biochemical Corp.). The micromethod of glucose determination described in the Glucostat methods manual was followed.

Prior to separating the sugars by TLC, a 25-ml. aliquot of the filtrates obtained from the takadiastase treatment of the residue following water extraction was passed through a glass column (1.9-cm. I.D.) containing 1.2 cm. of Amberlite IRA-400 (OH^- form) resin above 1.2 cm. of Amberlite IR-120 (H^+ form) resin. The flow rate was 2.5 ml. per minute. This effectively removed the protein and acetate and potassium ions from the filtrates. The column was washed five times with distilled water, and the entire solution and washings were concentrated by lyophilization. Sucrose and fructosans may be partially hydrolyzed by this procedure; therefore, the filtrates from direct takadiastase hydrolysis of the plant tissue could not be assayed.

All carbohydrate values are reported as percentage of dry weight (70° C.), and no allowances were made in the calculations for the water of hydrolysis for the polymerized carbohydrates.

RESULTS

Total Extraction with Various Solvents. The nonstructural carbohydrate values obtained in the filtrates after direct acid hydrolysis of timothy and alfalfa tissue, and in the filtrates before and after acid hydrolysis following water and takadiastase treatment of the tissues, are shown in Table I.

A very high nonstructural carbohydrate value (47.0%) was obtained from timothy by water extraction. An additional 1.2% was obtained when the residue from the water extraction was treated with takadiastase, giving a total of 48.2%. In contrast, water extraction of the alfalfa gave a carbohydrate value of 9.1%, and an additional 16.3% was obtained when the residue from the water extraction was treated with takadiastase.

Takadiastase alone hydrolyzed more nonstructural carbohydrate from both the timothy and alfalfa tissue than the sum of the carbohydrates from the water extraction and from takadiastase treatment of the residue following water extraction. The water filtrates apparently contained dextrins that were not hydrolyzed during 0.1*N* acid hydrolysis of these filtrates. This was indicated in a separate test when 1.2% more carbohydrate was obtained from timothy, and 3.2% more from alfalfa, when the water filtrate was treated with takadiastase and then was hydrolyzed with 0.1*N* H_2SO_4 than when hydrolyzed only with the acid.

Percentages obtained before and after acid hydrolysis of the filtrates obtained by direct takadiastase treatment of alfalfa and takadiastase treatment of the residues of timothy after water extraction were similar. This was not true with the direct enzyme treatment of timothy. Takadiastase was apparently degrading the glucose polymers to hexoses (alfalfa) but not the fructose polymers (timothy).

Table I. Total Nonstructural Carbohydrates (% Dry Weight) in Filtrates Obtained after Water, Acid, and Enzyme Treatment of Alfalfa and Timothy Tissue[a]

Treatment	% Carbohydrate: Before acid hydrolysis	% Carbohydrate: After acid hydrolysis[b]
	TIMOTHY	
Water	2.5 b	47.0 e
Takadiastase on residue	1.1 a	1.2 a
Total	3.6 c	48.2 f
Takadiastase	6.0 d	50.7 h
0.005*N* H_2SO_4		49.6 g
0.02*N* H_2SO_4		50.8 h
0.2*N* H_2SO_4		51.0 h
0.8*N* H_2SO_4		51.8 i
	ALFALFA	
Water	4.2 a	9.1 d
Takadiastase on residue	16.8 f	16.3 ef
Total	21.0 g	25.4 h
Takadiastase	30.2 k	29.2 j
0.005*N* H_2SO_4		7.2[c] b
0.02*N* H_2SO_4		8.4[c] c
0.2*N* H_2SO_4		15.8 e
0.8*N* H_2SO_4		28.2 i

[a] Any two means within a species followed by the same letter are not significantly different at the 1% level, and vice versa. The coefficient of variability was 1.9% for timothy and 4.6% for alfalfa based on 4 separate assays.
[b] Aliquots from the water and takadiastase filtrates were hydrolyzed with 0.1*N* H_2SO_4 for 15 minutes at 99° C. Filtrates from acid treatments were directly tested.
[c] A positive starch-iodine test was obtained.

The amount of nonstructural carbohydrate removed from timothy was slightly lower with 0.005*N* H_2SO_4 and slightly higher with 0.8*N* than with 0.02*N* and 0.2*N* H_2SO_4. Values from 0.005*N* H_2SO_4 were slightly higher than that obtained from water extraction plus that from takadiastase treatment of the residue following water extraction. Values with 0.02 and 0.2*N* H_2SO_4 were essentially the same as those of the direct takadiastase treatment of timothy after hydrolyzing with 0.1*N* H_2SO_4.

Small amounts of nonstructural carbohydrate were assayed from alfalfa following hot 0.005*N* and 0.02*N* H_2SO_4 hydrolysis. Values were less than with water extraction after hydrolyzing with 0.1*N* H_2SO_4. More carbohydrate was assayed with 0.2*N* acid, but less than with direct enzyme treatment. The 0.8*N* acid value was most comparable with that of direct enzyme treatment.

Some unhydrolyzed starch was removed from the alfalfa tissue with 0.005*N* and 0.02*N* acid, since a positive starch-iodine test was obtained in the filtrates. This test was negative with the 0.2*N* and 0.8*N* acid, indicating that any starch that was removed was hydrolyzed to glucose and/or short-chain dextrins.

Chromatographic Analysis. TLC was used to identify the sugars in the water filtrates and in the filtrates after enzyme treatment of the residue following water extraction, and both TLC and paper chromatography were used on all acid filtrates (Table II).

The water extract of timothy contained only small amounts of glucose, fructose, and sucrose. Most of the water-soluble carbohydrate remained at the origin on the chromatogram because of the lack of mobility of fructosans in the solvent system used. Water also extracted glucose,

Table II. Individual Carbohydrates (% Dry Weight) Separated by Chromatography from the Filtrates Obtained after Water, Acid, and Enzyme Treatment of Alfalfa and Timothy Tissue

Treatment	Raffinose	Maltose	Sucrose	Glucose	Fructose	Mannose	Galactose	Arabinose	Xylose	Total
				TIMOTHY						
Water	0	0	1.3	0.4	1.4	0	0	0	0	3.1
Takadiastase on residue	0	0	0	1.0	0	0	0	0	0	1.0
Total	0	0	1.3	1.4	1.4	0	0	0	0	4.1
0.005*N* H_2SO_4	0	...	0	1.5	45.4	0	0	1.3	0	48.2
0.02*N* H_2SO_4	0	...	0	1.6	45.9	0	0	1.6	0	49.1
0.2*N* H_2SO_4	...	...	0	1.4	44.9	0.7	0.3	1.6	0.8	49.7
0.8*N* H_2SO_4	...	...	0	1.8	39.5	1.8	0.6	1.3	6.0	51.0
				ALFALFA						
Water	2.0	3.3	4.4	0.7	0.2	0	0	0	0	10.6
Takadiastase on residue	0	0	0	15.5	0	0	0	0	0	15.5
Total	2.0	3.3	4.4	16.2	0.2	0	0	0	0	26.1
0.005*N* H_2SO_4	0	...	0	3.0	2.6	0	0	1.0	0	6.6
0.02*N* H_2SO_4	0	...	0	3.1	3.0	0	0.6	2.5	0	9.2
0.2*N* H_2SO_4	...	...	0	5.7	2.1	1.8	0.5	2.5	0.5	13.1
0.8*N* H_2SO_4	...	...	0	14.5	1.6	1.6	1.0	2.3	2.1	23.1

fructose, and sucrose from alfalfa, along with small amounts of raffinose, maltose, and nonmobile dextrins. Takadiastase treatment of the residue following water extraction of both timothy and alfalfa removed only glucose. Pentoses, which would have indicated takadiastase action on the hemicelluloses, were not present.

All acid strengths hydrolyzed the sucrose, raffinose, and fructosans from both timothy and alfalfa, and to different degrees the maltose and starch, and this increased the fructose and glucose contents.

Arabinose, along with glucose and fructose, was found in the 0.005*N* acid filtrates of timothy and alfalfa. Galactose was found in the 0.02*N* acid filtrates of alfalfa, and there was an increase in the arabinose content of both alfalfa and timothy. In the 0.2*N* filtrates of alfalfa, there was a marked increase in glucose, probably from the partial hydrolysis of starch. Also, mannose and xylose were present in the 0.2*N* filtrates of both alfalfa and timothy, and galactose appeared for the first time in timothy. These three sugars, along with arabinose, are constituents of hemicellulose.

No different sugars were found in the 0.8*N* acid filtrates than in the 0.2*N* filtrates. However, there was a considerable increase in the glucose content of alfalfa, due to the greater hydrolysis of starch. In both alfalfa and timothy there was an increase in xylose, most marked in timothy. Thus, hemicellulose was being hydrolyzed with the 0.8*N* acid. Concomitant with the large xylose increase in timothy, there was a decrease in fructose from 44.9 to 39.5%. Analysis of the 0.8*N* filtrates with resorcinol-HCl indicated that some fructose had been destroyed and converted to a furfural derivative.

The total amounts of nonstructural carbohydrate obtained by chromatographic analysis of the acid filtrates (Table II) were less than those obtained with the Shaffer-Somogyi reagent (Table I). Additional carbohydrates in the 0.8*N* acid filtrates, and to a lesser extent in the 0.2*N* filtrates, would have been obtained in the chromatographic analyses (Table II) if various di-, tri-, and larger oligosaccharides composed of hexoses and hexose-pentose mixtures containing a reducing group had been assayed.

DISCUSSION

Timothy contains some of the largest fructosan molecules found in plants (Smith and Grotelueschen, 1966), but they were effectively extracted from the tissue with water, and thus the summation values for fructose and glucose in the dilute acid extractions (Table II) and the total carbohydrate value from the water extraction after acid hydrolysis (Table I) were nearly equal. Water extracted maltose, raffinose, and dextrins from the alfalfa tissue in addition to fructose, glucose, and sucrose.

Analysis of the filtrates clearly indicated that fructosan-containing tissue, such as that of timothy, can be hydrolyzed with more dilute acid concentrations than starch-containing tissue, such as that of alfalfa. The most dilute acid concentration (0.005*N* H_2SO_4) appeared to hydrolyze the fructosans and sucrose completely. It also removed and hydrolyzed small amounts of arabinose from hemicellulose. The ease of hydrolysis of these compounds may be attributed to the fact that fructose and arabinose form polymers as coplanar, strained, furanoside moieties. The next higher acid solution (0.02*N* H_2SO_4) also effectively hydrolyzed the fructosans and sucrose, but additional arabinose was removed from the timothy and alfalfa tissue, and some galactose was removed from the alfalfa. Since no other hemicellulose constituent appeared other than arabinose, raffinose may have been the source of galactose. A dark-blue iodine test was obtained in the 0.005*N* and 0.02*N* acid filtrates of alfalfa and indicated that some unhydrolyzed starch had been removed from the tissue. The small increase in glucose over the water extraction is additional evidence that these acid concentrations were ineffective in hydrolyzing starch.

Some starch and hemicellulose were hydrolyzed to monomers beginning with the 0.2*N* acid concentration. Most of the increase in glucose in the alfalfa tissue can be attributed to some hydrolysis of starch. The appearance of xylose and mannose was the result of some hydrolysis of the hemicellulose. The 0.8*N* acid increased the amounts of both glucose and xylose that were removed from the tissue, indicating the difficulty of selectively hydrolyzing starch with an acid in the presence of hemicellulose.

In the alfalfa tissue, less than 60% of the possible glucose in the nonstructural carbohydrate appeared to have been hydrolyzed from alfalfa with 0.8*N* H_2SO_4, assuming the glucose content of the nonstructural carbohydrates to be about 25% (enzyme carbohydrate minus free fructose, fructose in sucrose, and fructose and galactose in raffinose). This recovery was low, since it also included the glucose hydrolyzed from hemicellulose sources. Starch and hemicellulose consist of pyranoside sugar moieties, except for arabinofuranoside moieties of hemicellulose. Thus, they were hydrolyzed to some degree by similar acid concentrations. However, it is probable that little of the cellulose was hydrolyzed, since its structure and bonding make it rather resistant to acid hydrolysis. Pectins also consist of pyranoside moieties. Thus, uronic acids may have been expected in the stronger acid hydrolysis. However, identification and analysis of uronic acids were not possible, as they would be precipitated in the neutralization procedure of the acid filtrates with $Ba(OH)_2$ and removed from the takadiastase filtrates by the purification procedure. As the individual sugar components obtained from TLC (Table II) nearly added up to the total nonstructural carbohydrate of the acid and takadiastase filtrates (Table I), the contribution of uronic acid–containing residues would seem to be comparatively small.

Concentrated acid solutions destroy fructose, and this was very apparent in the fructosan-containing timothy tissue. However, destruction was not noted until 0.2*N* acid was used. Unlike starch, fructosans can be hydrolyzed from the tissue with hot dilute acids—e.g., 0.005*N* H_2SO_4—in the presence of pyranoside moieties of hemicelluloses. Dilute acid concentrations may hydrolyze arabinofuranoside along with the fructofuranoside moieties, but the amount of arabinofuransoside was small in this study.

Takadiastase effectively hydrolyzed the glucosans, and this was most apparent in the alfalfa tissue where the starch content was high. However, takadiastase did not degrade the fructosans in timothy, and hydrolysis of the takadiastase filtrates with acid was necessary. The takadiastase preparation also had little hemicellulase or cellulase activity, since pentoses were not detected on the chromatograms of alfalfa or timothy tissue filtrates and there was virtually no activity on Whatman No. 40 filter paper. However, the takadiastase used was not a pure α- and β-amylase preparation. This was clearly demonstrated with the direct takadiastase treatment of the alfalfa tissue where the carbohydrates removed from the tissue were all hydrolyzed to reducing sugars. This indicated that takadiastase must have included sucrase, maltase, oligo-1,6-glucosidase, and possibly a melibiase. The difficulty of hydrolyzing melibiose, maltose, and starch with dilute acid makes takadiastase a very convenient enzyme preparation for the analysis of total nonstructural carbohydrates. The fact that takadiastase effectively hydrolyzed these compounds and that the acids did not, explains in part why direct takadiastase treatment gave higher total nonstructural carbohydrate values than water extraction followed by takadiastase treatment of the residue (Table I).

Previous work (Smith *et al.*, 1964) with tissue of fructosan-storing timothy and starch-storing alfalfa, and the present work with timothy, indicated that takadiastase and 0.2*N* H_2SO_4 gave similar total nonstructural carbohydrate values. However, seven of the 11 alfalfa samples in the previous work were low in total nonstructural carbohydrates, and thus were low in starch, increasing the probability of obtaining similar values. The present data indicate that similar values are less probable with tissue of high starch content, since the acid would only partially hydrolyze the starch and the takadiastase would completely hydrolyze it. Direct acid treatment also is very difficult in the presence of hemicellulose, whether the polysaccharide is starch or fructosan, as shown by the data with alfalfa. Partial hydrolysis of the hemicellulose by the acid solution tended to bring the total nonstructural carbohydrate values closer to those obtained from takadiastase treatment, even though the acid only partially hydrolyzed the starch.

Enzymatic analysis offers the most specific and accurate method for the determination of total nonstructural carbohydrates, especially with plant tissue containing large amounts of starch. With fructosan-containing tissue, acid hydrolysis will still be necessary following enzyme extraction to degrade the fructose polymers.

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