ALFALFA HEMICELLULOSE

Constitution of the Hemicellulose of Alfalfa (*Medicago sativa*). Hydrolysis of Hemicellulose and Identification of Neutral and Acidic Components

D. V. MYHRE and FRED SMITH

Department of Agricultural Biochemistry, University of Minnesota, St. Paul 1, Minn.

The work was carried out to determine whether there is a relationship between nutritional value and structure of the hemicelluloses of grasses and legumes. The hemicellulose of alfalfa (*Medicago sativa* var. Ranger) gives upon hydrolysis a mixture of sugars composed of L-arabinose (12.0%), D-xylose (67.3%), D-galactose (8.1%), D-glucose (8.1%), and L-rhamnose (4.5%), with a mixture of seven acidic components. Five of these acids are oxalic acid, D-galacturonic acid, 4-O-methyl-D-glucuronic acid, 2-O-(4-O-methyl-α-D-glucosyluronic acid)-D-xylose (I), and O-4-O-methyl-α-D-glucosyluronic acid-(1 → 2)-O-β-D-xylopyranosyl-(1 → 4)-β-D-xylose (Vb). The other two appear to be D-galactosyluronic acid D-xylose and D-galactosyluronic acid D-galactose. The results may have some bearing on the biochemistry of animal nutrition and be useful to the plant breeder.

CERTAIN FORAGE CROPS, notably the legumes, have long been known to be more nutritious than the grasses. Because differences in hemicellulose composition and structure may be responsible for some of the observed differences in feed value, a series of investigations has been initiated to ascertain the composition and structure of the hemicelluloses of certain selected grasses and legumes.

This communication deals with the preliminary results of studies on the constitution of the hemicellulose of alfalfa (*Medicago sativa* var. Ranger), one of the major legume crops of the midwestern area of the United States.

Experimental

Unless stated otherwise, all evaporations were carried out in vacuo at 40° to 50° C. (bath temperature).

The solvents used in chromatography were A, pyridine–ethyl acetate–water (1:2.5:3.5); B,*n*-butyl alcohol–pyridine–water (6:4:3); C,*n*-butyl alcohol–acetic acid–water (2:1:1); D, butanone-water azeotrope; E, *n*-butyl alcohol–ethyl alcohol–water (4:1:5).

The cation resin, Amberlite IR-120 (H^+ form), and the anion resin, Duolite A-4 (OH^- form), were used throughout unless specified otherwise.

Isolation of Alfalfa Hemicellulose. Alfalfa hay (400 grams) was extracted with hot 80% aqueous methanol for 2 hours and then washed with distilled water. The wet residue was heated with 8 liters of water which contained 188 grams of sodium chlorite and 40 grams of acetic acid for 5 hours at 75° to 80° C. to remove the lignin (*27*). The white holocellulose was washed with distilled water until free of chloride ion and treated with 4 liters of 10% sodium hydroxide for 4 hours at 25° C. with occasional stirring. After filtration, the residue was heated with 2 liters of 10% sodium hydroxide for 3 hours at 80° to 90° C. and filtered again. The combined filtrates were acidified with acetic acid and 4 volumes of ethyl alcohol were added to precipitate the hemicellulose. The latter was washed successively with ethyl alcohol, diethyl ether, and petroleum ether (boiling point 35° to 60° C.) and dried in vacuo. The yield of crude hemicellulose was 49.6 grams or 12.4% of the original alfalfa hay.

Determination of Neutral Equivalent of Hemicellulose. A portion of the hemicellulose (1.5 grams) was dissolved in 5% sodium hydroxide; a small amount of insoluble residue was removed by centrifugation and discarded. The supernatant solution was acidified with 1*N* hydrochloric acid and dialyzed against distilled water until chloride ion was absent from the dialyzate. The hemicellulose was precipitated with ethyl alcohol and dried as described above.

Excess 0.02*N* sodium hydroxide (25 ml.) was added to 0.2946 gram of the hemicellulose and the excess alkali was back-titrated with 14.15 ml. of 0.0203*N* hydrochloric acid. A blank experiment carried out at the same time required 24.63 ml. of 0.0203*N* hydrochloric acid. Found: equivalent weight, 1384, 1380.

Hydrolysis of Alfalfa Hemicellulose. ISOLATION AND IDENTIFICATION OF NEUTRAL SUGAR COMPONENTS. The hemicellulose (10 grams) was heated with 100 ml. of 1*N* sulfuric acid on a boiling water bath for 9 hours. The solution was neutralized with barium carbonate, centrifuged, and passed successively through the cation and anion resins. The organic acids were absorbed on the latter. The solution containing the neutral sugars was evaporated to dryness, giving 8.2 grams of a thick sirup.

A portion of the mixed neutral sugars (1.8 grams) was resolved on a cellulose column with solvent B in the usual manner, the effluent from the column being collected (6 ml. every 15 minutes) in test tubes with an automatic fraction collector. The appropriate fractions were combined and the solvent was evaporated to give the five sugars listed in Table I, each recrystallized from methanol.

DETERMINATION OF RATIO OF NEUTRAL SUGARS. A portion of the neutral sugar mixture (22.7 mg.) was applied to Whatman No. 1 filter paper (8 × 22 inches). After irrigation with solvent A, three strips were cut from the paper (one in the center and one from each side) and sprayed with *p*-anisidine trichloroacetate in order to locate the position of the sugars. Each sugar was eluted from the appropriate section of the paper with water: the D-xylose with 10 ml., the L-arabinose with 10 ml., the D-glucose with 5 ml., the D-galactose with 5 ml., and the L-rhamnose with 10 ml. Each solution was filtered through glass wool. The D-xylose eluate was diluted with water 50 times, the L-arabinose 12.5 times, the D-glucose 12.5 times, the D-galactose 25 times,

Table I. Identification of Sugars in Hydrolyzate of Alfalfa Hemicellulose

Sugar	%	M.P. and Mixed M.P., ° C.	$[\alpha]_D^{25}$ (Equilibrium Value in Water)
D-Xylose	67.3	144–45	+18.3° (*c*, 1)[a]
L-Arabinose	12.0	158–59	+102° (*c*, 1)
D-Glucose	8.1	146	+52° (*c*, 5)
D-Galactose	8.1	166–67	+72° (*c*, 1)
L-Rhamnose	4.5	92–3.5 monohydrate	+8.0° (*c*, 5)

[a] Here and elsewhere *c* refers to concentration of solute in grams per 100 ml. of solution.

and the L-rhamnose 12.5 times. The concentration of each sugar was determined on 2 ml. of each of the diluted solutions by the phenol–sulfuric acid method (*6*). The results are recorded in Table I.

GRADED HYDROLYSIS OF HEMICELLULOSE AND ISOLATION OF MIXTURE OF URONIC ACID COMPONENTS. To improve the yield of the mono-*O*-methylaldotriouronic acid (Vb), 30 grams of the hemicellulose was heated with 0.5*N* sulfuric acid on a boiling water bath for 3 hours. The insoluble residue was removed by centrifugation and retreated with 0.5*N* sulfuric acid as before. Likewise the residue from this second hydrolysis was removed and heated with 1*N* sulfuric acid for 3 hours. All three hydrolyzates were combined, neutralized with barium carbonate, and centrifuged, and the supernatant solution was passed through the cation and anion resins. The latter was washed with water until free of neutral sugars (negative Molisch test). The acidic components were eluted from the anion resin with 1*N* sodium hydroxide and recovered by passing the alkaline eluate through the cation resin.

Paper chromatographic analysis of the mixture of uronic acids with solvent C indicated five components; the one having the greatest R_F value, 0.49, corresponded to 4-*O*-methyl-D-glucuronic acid, followed by a mono-*O*-methylaldobiouronic acid (R_F, 0.41), D-galacturonic acid (R_F, 0.31), a mono-*O*-methylaldotriouronic acid (R_F, 0.24), and a mixture of two oligouronic acids (R_F, 0.16).

Isolation of Oxalic Acid. The addition of calcium acetate to an aqueous solution of the acid mixture gave a small amount of white precipitate, which was removed by filtration and dissolved in dilute hydrochloric acid. Calcium ions were removed with the cation resin and the effluent was evaporated to dryness. A portion of the residue sublimed at 100° to 110° C. to give oxalic acid dihydrate, recognized by melting point and mixed melting point 100° to 101° C., and by the fact that it decolorized acidified (1*N* sulfuric acid) potassium permanganate at 60° C.

The amount of oxalic acid produced during hydrolysis of the hemicellulose was determined as follows: The hemicellulose (5 grams) from freshly harvested, air-dried alfalfa was isolated as described above and heated with 70 ml. of 1*N* hydrochloric acid on a boiling water bath for 6 hours. The solution was treated with just less than the stoichiometric amount of barium carbonate, followed by an excess of barium acetate. The white precipitate of barium oxalate was removed by centrifugation, washed with 10 ml. of water, and dissolved in dilute hydrochloric acid, and the barium ions were removed with the cation resin. The effluent was made alkaline with ammonium hydroxide and then acidified with acetic acid, after which an excess of 1*N* calcium chloride was added. The white precipitate of calcium oxalate was separated by centrifugation, washed with water, acetone, and diethyl ether, and dried; yield, 0.215 gram. This corresponds to 3% oxalic acid in the hemicellulose.

The calcium oxalate was dissolved in dilute hydrochloric acid and the free oxalic acid dihydrate was isolated by evaporation and sublimation as described above; melting point and mixed melting point 100° to 101° C.

Separation and Identification of Uronic Acids. An attempt to separate the uronic acid mixture by displacement of the acids from Amberlite IRA-400 resin (acetate form) with increasing concentrations of acetic acid (*2*, *5*, *25*) gave only a partial separation.

The uronic acid mixture was therefore separated on a cellulose column with solvent C using an automatic fraction collector adjusted to collect about 7 ml. per fraction every 20 minutes. Tubes 25 and 33 contained the 4-*O*-methyl-D-glucuronic acid; tubes 38 to 48, the mono-*O*-methylaldobiouronic acid (I); tubes 60 to 72, galacturonic acid; tubes 70 to 82, the mono-*O*-methylaldotriouronic acid (Vb); and tubes 140 to 155, the oligouronic acid components. The mono-*O*-methylaldotriouronic acid (Vb) crystallized spontaneously after evaporation of the solvent and exposure to the atmosphere.

IDENTIFICATION OF D-GALACTURONIC ACID. The uronic acid with an R_F value corresponding to that of galacturonic acid gave a brick red precipitate when heated with an aqueous solution of basic lead acetate (*7*).

The fraction crystallized spontaneously and after recrystallization from aqueous ethyl alcohol afforded D-galacturonic acid monohydrate; melting point and mixed melting point 156° C. (with decomposition) (during heating the crystals turned yellow at 115° and red at 130°), $[\alpha]_D^{22}$ + 95° changing to +51° after 24 hours (equilibrium value, *c*, 3.4 in water).

IDENTIFICATION OF 4-*O*-METHYL-D-GLUCURONIC ACID. The 4-*O*-methyl-D-glucuronic acid (28 mg.) obtained by column chromatography was refluxed with 1% methanolic hydrogen chloride for 5 hours, neutralized with silver carbonate, filtered, and evaporated to a sirup. A solution of the sirup in methanol was saturated with ammonia gas at 0° C. and allowed to stand overnight at 3° C., after which the solution was evaporated to a sirup at room temperature. Nucleation of the sirup with methyl 4-*O*-methyl-α-D-glucosiduronamide (*21*) provided crystals with melting point 195° to 205° C. Examination of the crystals under the microscope revealed the presence of both alpha and beta isomers, which are easily distinguished because the latter forms platelets and the former small needles. The methyl 4-*O*-methyl-α-D-glucosiduronamide was obtained by triturating the mixture with ethyl alcohol; melting point and mixed melting point 235° to 236° C. after recrystallization from methanol.

IDENTIFICATION OF 2-*O*-(4-*O*-METHYL-α-D-GLUCOSYLURONIC ACID)-D-XYLOSE (I). The mono-*O*-methylaldobiouronic acid (I) (73 mg.), which showed $[\alpha]_D^{26}$ + 95° (*c*, 2.3 in water) and equivalent weight 350 (calculated for $C_{12}H_{20}O_{11}$, 340), was heated with methanol containing hydrogen chloride (8%) in a sealed tube at 110° to 115° C. for 11 hours. The solution was neutralized with silver carbonate, filtered, and evaporated to a sirup. The sirup was dissolved in methanol which was saturated with ammonia gas at 0° C. and the mixture was allowed to stand overnight at 3° C. After evaporation of the methanol in vacuo at room temperature, the amide crystallized. A small amount of ethyl alcohol was added and the sirupy mother liquor was withdrawn. The crystalline residue was triturated with ethyl alcohol and recrystallized from methanol to give methyl 4-*O*-methyl-α-D-glucosiduronamide, melting point and mixed melting point 235° to 236° C., $[\alpha]_D^{25}$ + 146° (*c*, 1.6 in water) (*21*).

The mother liquor from the previous experiment which contained the methyl xyloside was freed from solvent and heated with 1*N* sulfuric acid on a boiling water bath for 8 hours. The solution was neutralized with barium carbonate, centrifuged, and passed successively through the cation and the anion resins. Evaporation of the eluate gave a sirup which crystallized on trituration with

methanol. Recrystallization of the product from methanol gave D-xylose; melting point and mixed melting point 144° to 145° C., $[\alpha]_D^{25}$ + 19.9° (equilibrium value, *c*, 4 in water).

METHYL 2-*O*-(2,3,4,6-TETRA-*O*-METHYL-α-D-GLUCOSYL)-3,4-DI-*O*-METHYL-D-XYLOSIDE (III). The mono-*O*-methylaldobiouronic acid (I) (0.54 gram) was refluxed with 1% methanolic hydrogen chloride for 6 hours. The solution was neutralized with silver carbonate, filtered, and evaporated to give methyl 2-*O*-[methyl-(4-*O*-methyl-α-D-glucosyl)uronate]-D-xylopyranoside as a sirup.

A portion of the methyl 2-*O*-[methyl-(4-*O*-methyl-α-D-glucosyl)uronate]-D-xylopyranoside (334 mg.) was shaken with 10 ml. of dimethylformamide, 3 ml. of methyl iodide, and 1 gram of silver oxide for 24 hours (*13*). The mixture was filtered and the residue was washed with 2 ml. of dimethylformamide and 15 ml. of chloroform. An excess of chloroform was added to the filtrate and the silver salts which precipitated were removed by filtration. The filtrate was washed with a small volume of aqueous potassium cyanide to remove the last traces of silver salts, dried over anhydrous magnesium sulfate, and concentrated to a sirup. The sirup was further methylated by refluxing with 8 ml. of methyl iodide and 0.5 gram of silver oxide for 18 hours, after which the mixture was filtered and the methyl iodide was evaporated, leaving 0.34 gram of methyl 2-*O*-[methyl-(2,3,4-tri-*O*-methyl-α-D-glucosyl)uronate]-3,4-di-*O*-methyl-D-xyloside (II) as a sirup.

A portion (0.20 gram) of methyl 2-*O*-[methyl-(2,3,4-tri-*O*-methyl-α-D-glucosyl) uronate]-3,4-di-*O*-methyl-D-xyloside (II), obtained above, was heated with 5 ml. of 1*N* sulfuric acid on a boiling water bath for 11 hours. The brownish solution was passed through the anion resin to remove the mineral acid and uronic acid. Concentration of the effluent provided only 26 mg. of sirup, which indicated that hydrolysis was not complete. The acid component was eluted from the anion resin with 1*N* sodium hydroxide, passed through the cation resin, and then neutralized with barium carbonate. The barium uronate was converted to the free acid with cation resin and the effluent was concentrated. The sirup was heated with 13% methanolic hydrogen chloride in a sealed tube at 110° to 115° C. for 12 hours. The solution was neutralized with silver carbonate, filtered, and evaporated to a sirup. The ester was saponified with 0.5*N* sodium hydroxide at 50° C. for 1 hour. The solution was passed first through the cation resin, then the anion resin, and the effluent evaporated to give 18 mg. of methyl 3,4-di-*O*-methyl-D-xyloside.

The methyl 2,3,4-tri-*O*-methyl-D-glucosiduronic acid was isolated from the anion resin in the usual manner and was refluxed with 2% methanolic hydrogen chloride for 6 hours. The solution was neutralized with silver carbonate, filtered, and evaporated to a sirup. A solution of the sirup in methanol was saturated with ammonia gas at 0° C. and allowed to stand at 3° C. for 24 hours. After evaporation of the methanolic ammonia, the amide so formed was recrystallized from ethyl alcohol–diethyl ether, giving methyl 2,3,4-tri-*O*-methyl-D-glucosiduronamide (*20*) as colorless needles; melting point and mixed melting point 184° to 185° C., $[\alpha]_D^{25}$ + 143° (*c*, 1 in water).

The methyl 3,4-di-*O*-methyl-D-xyloside was hydrolyzed with 1*N* sulfuric acid by heating in a sealed tube in a boiling water bath for 11 hours. The solution was neutralized with barium carbonate and centrifuged and the supernatant was evaporated to dryness. The 3,4-di-*O*-methyl-D-xylose extracted from the residue with chloroform was combined with that obtained by sulfuric acid hydrolysis of methyl 2-*O*-[methyl-(2,3,4-tri-*O*-methyl-α-D-glucosyl)-uronate]-3,4-di-*O*-methyl-D-xyloside (II), purified by sheet paper chromatography using solvent D, and combined with the 3,4,-di-*O*-methyl-D-xylose obtained in the following experiment for the purpose of identification.

A solution of 0.14 gram of the methyl 2-*O*-[methyl-(2,3,4-tri-*O*-methyl-α-D-glucosyl)uronate]-3,4-di-*O*-methyl-D-xyloside (II) in 5 ml. of dry tetrahydrofuran was added dropwise with stirring to a solution of 0.2 gram of lithium aluminum hydride in 5 ml. of tetrahydrofuran (*1*, *15*). The mixture was refluxed for 1 hour and cooled to room temperature. The excess lithium aluminum hydride was decomposed by the dropwise addition of ethyl acetate. After addition of 30 ml. of water, the mixture was acidified with dilute hydrochloric acid and extracted with chloroform (4 × 20 ml.). The combined chloroform extracts were dried over anhydrous magnesium sulfate together with a small amount of sodium carbonate to neutralize any trace of hydrochloric acid. The chloroform solution was filtered and evaporated to a sirup which was methylated twice with 8 ml. of methyl iodide and 0.2 gram of silver oxide, giving 120 mg. of methyl 2-*O*-(2,3,4,6-tetra-*O*-methyl-α-D-glucosyl)-3,4-di-*O*-methyl-D-xyloside (III) as a mobile sirup having $[\alpha]_D^{25}$ + 111° (*c*, 1.2 in methanol). Analysis. Calcd. for $C_{18}H_{34}O_{10}$, OMe 52.9. Found: OMe, 53.1.

A solution of methyl 2-*O*-(2,3,4,6-tetra-*O*-methyl-α-D-glucosyl)-3,4-di-*O*-methyl-D-xyloside (III) in 5 ml. of 1*N* sulfuric acid was heated on a boiling water bath for 11 hours. The solution was neutralized with barium carbonate, centrifuged, and concentrated to dryness. The residue was extracted with ethyl alcohol and, after treatment with a small amount of charcoal, evaporation of the extract gave 103 mg. of a sirupy mixture of 2,3,4,6-tetra-*O*-methyl-D-glucose and 3,4-di-*O*-methyl-D-xylose, $[\alpha]_D^{23}$ + 64° (*c*, 2 in methanol).

The two sugars were separated on filter paper (Whatman No. 3MM) with solvent D as before. The component (29 mg.) with an R_F value corresponding to that of 3,4-di-*O*-methyl-D-xylose which had $[\alpha]_D^{22}$ + 22° (*c*, 3 in methanol) was refluxed for 4 hours with 5 ml. of ethyl alcohol containing 50 mg. of aniline. After evaporation of the ethyl alcohol and excess aniline, the product crystallized spontaneously. Recrystallization from ethyl acetate–petroleum ether (boiling point 35° to 60° C.) gave *N*-phenyl-3,4-di-*O*-methyl-D-xylosylamine, melting point 122° to 123° C. (*12*), $[\alpha]_D^{24}$ − 105° (*c*, 0.5 in ethyl acetate).

The mother liquor from the crystallization of the *N*-phenyl-3,4-di-*O*-methyl-D-xylosylamine was treated with charcoal in aqueous ethyl alcohol and filtered. A small amount of cation resin was added to the filtrate and the mixture was heated at 70° C. for 1 hour (*10*). The resin was removed by filtration and washed with ethyl alcohol. Evaporation of the combined filtrate and washings provided 15 mg. of sirupy 3,4-di-*O*-methyl-D-xylose, which was dissolved in 1 ml. of water and oxidized with 0.1 ml. of bromine in the dark at room temperature for 4 days. At the end of this time the excess bromine was removed by aeration and the solution was neutralized with silver carbonate, filtered, and passed through the cation resin. The acidic effluent was evaporated. Lactonization by heating at 85° C. in vacuo afforded 3,4-di-*O*-methyl-D-xylono-δ-lactone which readily sublimed; melting point and mixed melting point 65.5° to 66.5° C., $[\alpha]_D^{24}$ − 45° changing to −14.5° (equilibrium value *c*, 1.4 in water) (*11*).

The component derived from methyl 2-*O*-(2,3,4,6-tetra-*O*-methyl-α-D-glucosyl)-3,4-di-*O*-methyl-D-xyloside (III) with an R_F value corresponding to that of 2,3,4,6-tetra-*O*-methyl-D-glucose was treated with a small amount of charcoal in methanol. Evaporation of the solvent gave crystalline 2,3,4,6-tetra-*O*-methyl-D-glucose; melting point and mixed melting point 86° to 88° C., $[\alpha]_D^{24}$ + 81° (equilibrium value, *c*, 2.3 in water) after recrystallization from diethyl ether–petroleum ether (boiling point 35° to 60° C.).

METHYL 2-*O*-[METHYL-(4-*O*-METHYL-α-D-GLUCOSYL)URONATE]-α-D-XYLOPYRANOSIDE TETRAACETATE (IV) (*24*). The methyl 2-*O*-[methyl-(4-*O*-methyl-α-D-glucosyl)uronate]-D-xyloside (II) (200 mg.) was heated at 90° to 95° C.

with 0.4 gram of anhydrous sodium acetate and 15 ml. of acetic anhydride for 4 hours. The excess acetic anhydride was evaporated and the residue was shaken with a mixture of 20 ml. of chloroform and 20 ml. of water. The aqueous layer was separated and extracted four times with 20-ml. portions of chloroform. The combined chloroform extracts were dried over anhydrous magnesium sulfate and evaporated to give 288 mg. of a sirup which crystallized upon trituration with ethyl alcohol. Recrystallization from acetone–ethyl alcohol gave methyl 2-*O*-[methyl-(2,3-di-*O*-acetyl-4-*O*-methyl-α-D-glucosyl)uronate]-3,4-di-*O*-acetyl-α-D-xyloside (IV) (*24*); melting point and mixed melting point 200° to 202° C., $[\alpha]_D^{26}$ + 98° (*c*, 1.4 in chloroform). Analysis. Calcd. for $C_{22}H_{32}O_{15}$: C, 49.25; H, 6.02; OMe, 17.3. Found: C, 49.50; H, 5.74; OMe, 18.4.

Identification of *O*-4-*O*-Methyl-α-D-glucosyluronic acid-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-β-D-xylose (V). Reduction and Identification of Hydrolysis Products. Recrystallization of the crystalline mono-*O*-methylaldotriuronic acid (Vb) from aqueous methanol gave crystals with melting point 178° C., undepressed when mixed with the mono-*O*-methylaldotriouronic acid isolated from the wood of western hemlock (*Tsuga heterophylla*) (*8*), $[\alpha]_D^{23}$ + 45° changing to +52° (equilibrium value, *c*, 2.2 in water). Analysis. Calcd. for $C_{17}H_{28}O_{15}.3H_2O$: C, 38.8; H, 6.46; OMe, 5.89, equivalent weight, 526. Found: C, 38.95; H, 6.54; OMe, 7.6; equivalent weight, 540.

The mono-*O*-methylaldotriouronic acid (Vb) (108 mg.) was allowed to stand with 1% methanolic hydrogen chloride at room temperature for 1 week, after which time paper chromatography (solvent C; spray reagent, *p*-anisidine) indicated that glycoside formation was complete. The solution was neutralized with silver carbonate, filtered, and concentrated to give methyl [methyl-*O*-(4-*O*-methyl-α-D-glucosyl)uronate-(1→2)-*O*-β-D-xylopyranosyl-(1→4)]-D-xyloside (VI) as a sirup.

A solution of 62 mg. of VI in 10 ml. of tetrahydrofuran was added dropwise with stirring to a solution of 0.2 gram of lithium aluminum hydride in 5 ml. of tetrahydrofuran (*1*). After the mixture had been refluxed for 2.5 hours, the excess lithium aluminum hydride was decomposed with ethyl acetate and the reaction mixture acidified with acetic acid. The mixture was evaporated to dryness and the residue acetylated by heating at 85° to 95° C. with 0.2 gram of anhydrous sodium acetate and 8 ml. of acetic anhydride for 4 hours. The excess acetic anhydride was evaporated and the residue shaken with a mixture of 25 ml. of 0.5*N* hydrochloric acid and 25 ml. of chloroform. The aqueous layer was extracted four times with 15-ml. portions of chloroform and the combined chloroform extracts were dried over anhydrous sodium sulfate together with a small amount of anhydrous sodium carbonate. Evaporation of the chloroform gave 109 mg. of the acetylated trisaccharide, $[\alpha]_D^{26}$ + 44° (*c*, 2.1 in chloroform), which did not crystallize.

The heptaacetate obtained in the previous experiment was deacetylated (*28*) by dissolving it in anhydrous methanol which contained a trace of sodium and allowing the solution to stand overnight at room temperature. Evaporation of the methanol gave 62 mg. of the sirupy methyl glycoside of a trisaccharide which had $[\alpha]_D^{25}$ + 52° (*c*, 1.25 in water). The sirup was heated for 8.5 hours with 5 ml. of 0.5*N* sulfuric acid on a boiling water bath until a constant rotation of $[\alpha]_D^{25}$ + 33° (*c*, 1.25 in 0.5*N* sulfuric acid) had been reached. The hydrolyzate was passed through the anion resin and the effluent was evaporated to 53 mg. of a sirup.

The sugars were separated by chromatography on two pieces (9 × 22 inches) of Whatman No. 1 filter paper with solvent E. The component (18 mg.) with an R_F value corresponding to that of 4-*O*-methyl-D-glucose was heated at 85° C. with 2 ml. of water, 0.2 ml. of acetic acid, and 0.1 ml. of phenylhydrazine for 2 hours (*19*, *21*). The crystalline osazone which separated from solution on cooling was recrystallized from aqueous acetone to give 4-*O*-methyl-D-glucosephenylosazone; melting point and mixed melting point 157° to 158° C., $[\alpha]_D^{25}$ − 35° changing to ±0° (equilibrium value, *c*, 0.55 in ethyl alcohol).

The component (30 mg.) with an R_F value corresponding to that of xylose was dissolved in methanol and treated with a small amount of charcoal. Filtration, evaporation, and recrystallization from methanol gave D-xylose; melting point and mixed melting point 144° to 145° C., $[\alpha]_D^{27}$ + 18.4° (equilibrium value, *c*, 3.1 in water).

Methyl-*O*-[2,3,4,6-Tetra-*O*-methyl-α-D-glucosyl-(1→2)-*O*-3,4-di-*O*-methyl-β-D-xylosyl-(1→4)]-2,3-di-*O*-methyl-D-xyloside (VII). The methyl ester methyl glycoside (VI) of the mono-*O*-methylaldotriouronic acid (Vb) (46 mg.) was methylated by the Kuhn technique (*13*) as described above for the mono-*O*-methylaldobiouronic acid. The sirupy product was methylated twice with 7 ml. of methyl iodide and 0.3 gram of silver oxide by refluxing overnight, methanol being added in the first treatment to dissolve the sirup.

The sirupy methylated product was dissolved in 5 ml. of tetrahydrofuran and the solution was added dropwise with stirring to a solution of 0.15 gram of lithium aluminum hydride in 5 ml. of tetrahydrofuran. The mixture was refluxed for 2 hours, cooled, and treated with 50 ml. of water, the water in the initial stage being added dropwise until the lithium aluminum hydride had been destroyed. The solution was acidified with dilute hydrochloric acid and the partially methylated trisaccharide extracted with chloroform (4 × 20 ml.). The combined chloroform extracts were dried in the usual manner and evaporated to a sirup. The latter was refluxed overnight with Purdie reagent (5 ml. of methyl iodide, 0.3 gram of silver oxide), filtered, and evaporated to give the nona-*O*-methyl derivative (VII) as a sirup which was heated with 1*N* sulfuric acid on a boiling water bath for 8 hours. The hydrolyzate was passed through the anion resin to remove the mineral acid and the effluent was concentrated to a sirup. Paper chromatography with solvent D and *p*-anisidine spray reagent revealed two spots, one corresponding to 2,3,4,6-tetra-*O*-methyl-D-glucose and the other to a di-*O*-methylxylose. Paper electrophoresis (*4*) (0.1*M* borate buffer and *p*-anisidine–phosphoric acid spray reagent) of the di-*O*-methylxylose component, separated from the 2,3,4,6-tetra-*O*-methyl-D-glucose by paper chromatography using solvent D, revealed two components, one giving a deep purple color corresponding to 2,3-di-*O*-methyl-D-xylose and the other a somewhat less intense pinkish red color corresponding to 3,4-di-*O*-methyl-D-xylose. The mixture of the two di-*O*-methylxyloses was boiled for 3 hours with 1 ml. of ethyl alcohol containing 20 mg. of aniline, and the solution was treated with a small amount of charcoal, filtered, and evaporated to a sirup. A drop of ethyl acetate was added to the sirup and the mixture nucleated with the aniline derivative of 2,3-di-*O*-methyl-D-xylose. Recrystallization of the product from ethyl acetate gave *N*-phenyl-2,3-di-*O*-methyl-D-xylosylamine, melting point and mixed melting point 125° to 127° C. $[\alpha]_D^{26}$ − 173° (*c*, 0.2 in ethyl acetate).

Methanolysis of *O*-4-*O*-Methyl-α-D-glucosyluronic acid-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-β-D-xylose. The mono-*O*-methylaldotriouronic acid (Vb) (82 mg.) was refluxed with 3% methanolic hydrogen chloride for 20 hours. The solution was neutralized with silver carbonate, filtered, and evaporated to a sirup, which was saponified with 0.5*N* sodium hydroxide at 50° C. for 1 hour and passed through the cation and anion resins. The effluent containing the neutral component was evaporated to give methyl β-D-xylopyranoside; melting point and mixed melting point 155° to 157° C. (after recrystallization from ethyl alcohol).

The acid component was removed from the anion resin with 0.5*N* sodium hydroxide and converted to the free acid with the cation resin. Evaporation

of the effluent gave 51 mg. of a sirup which was refluxed with 2% methanolic hydrogen chloride for 4 hours; the solution was cooled, neutralized with silver carbonate, filtered, and evaporated to give methyl 2-*O*-[methyl-(4-*O*-methyl-α-D-glucosyl)uronate]-D-xylopyranoside as a sirup. This methyl ester, methyl glycoside of 2-*O*-(4-*O*-methyl-α-D-glucosyluronic acid)-D-xylose (I), was acetylated with acetic anhydride and sodium acetate by the procedure described above for the mono-*O*-methyl-aldobiouronic acid to give methyl 2-*O*-[methyl-(2,3-di-*O*-acetyl-4-*O*-methyl-α-D-glucosyl) uronate]-3,4-di-*O*-acetyl-α-D-xyloside (IV), melting point and mixed melting point 200° to 201° C. (after recrystallization from ethyl alcohol), $[\alpha]_D^{24}$ + 95° (*c*, 1 in chloroform).

Discussion

The alfalfa hemicellulose has been shown to give, upon acid hydrolysis, a mixture of L-arabinose (12.0%), D-xylose (67.3%), D-galactose (8.1%), D-glucose (8.1%), and L-rhamnose (4.5%), with a mixture of seven acidic components. Five of these acids have been identified as oxalic acid, D-galacturonic acid, 4-*O*-methyl-D-glucuronic acid which probably corresponds to the methoxyglycuronic acid previously reported (*9, 18*), 2-*O*-(4-methyl-α-D-glucosyluronic acid)-D-xylose (I), and *O*-4-*O*-methyl-α-D-glucosyluronic acid-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-β-D-xylose (Vb). The other two appear from preliminary studies to be D-galactosyluronic acid-(1→?)-D-xylosyl-(1→?)-D-xylose and D-galactosyluronic acid (1→?)-D-galactose.

The presence of 4.5% of L-rhamnose in the hemicellulose was of interest, in view of the fact that little or no rhamnose had been found in the hemicellulose of brome grass (*Bromus inermis*), timothy (*Phleum pratense*), or prairie grass (*Buchloe dactyloides*). The relatively high content of L-rhamnose appears to be a characteristic of the legumes, as the hemicelluloses of sweet clover (*Melilotus alba*), red clover (*Trifolium pratense*), alsike clover (*Trifolium hybridum*), bird's-foot trefoil (*Lotus corniculatus*), and Dutch clover (*Trifolium repens*) all contain a considerable proportion of L-rhamnose (*17*). Other studies (*17*) have shown that the hemicelluloses in the leaves of the elm (*Ulmus americana*), honey locust (*Gleditsia triacanthos*), maple (*Acer saccharum*), and oak (*Quercus alba*) contain significant amounts of L-rhamnose.

The alfalfa hemicellulose was isolated as a white amorphous powder ($[\alpha]_D$ − 35° in 1*N* sodium hydroxide) from alfalfa (*Medicago sativa* var. Ranger) hay, harvested in Minnesota, in the usual manner by chlorite delignification followed by extraction with dilute sodium hydroxide. Acid hydrolysis of the hemicellulose followed by the use of exchange resins afforded the mixture of the neutral sugars referred to above—L-arabinose, D-xylose, D-galactose, D-glucose, and L-rhamnose—all of which were obtained crystalline, and a mixture of the seven acidic components. The oxalic acid, the source of which is unknown, was separated as the calcium salt and identified as the dihydrate. The remaining uronic acid mixture was resolved by chromatography on a cellulose column and shown to consist of D-galacturonic acid, 4-*O*-methyl-D- glucuronic acid, 2-*O*-(4-*O*-methyl-α-D-glucosyluronic acid)-D-xylose (I), *O*-4-*O*-methyl-α-D-glucosyluronic acid-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-β-D-xylose (Vb), and two unidentified oligouronic acids.

The D-galacturonic acid, which was identified as the crystalline monohydrate (*14*), does not appear to be derived from pectin, because the two unidentified oligouronic acids themselves were shown to contain galacturonic acid and because the method used for isolating the hemicellulose precluded the possibility of contamination with pectin.

The 4-*O*-methyl-D-glucuronic acid was converted with methanolic hydrogen chloride into the methyl ester methyl glycosides which upon treatment with ammonia afforded the characteristic crystalline α- and β- forms of methyl 4-*O*-methyl-D-glucosiduronamide (*21, 26*).

The 2-*O*-(4-*O*-methyl-α-D-glucosyluronic acid)-D-xylose (I), which was liberated by prolonged hydrolysis of the hemicellulose, was identified by reason of the fact that, upon methanolysis, it yielded methyl (4-*O*-methyl-α-D-glucosid) uronate, identified as the corresponding amide (*21, 26*), and methyl D-xyloside, identified after hydrolysis as crystalline D-xylose. Moreover, methylation of the methyl ester methyl glycoside of 2-*O*-(4-*O*-methyl-α-D-glucosyluronic acid)-D-xylose, prepared by boiling with 1% methanolic hydrogen chloride, followed by lithium aluminum hydride reduction (*1, 15*) and methylation, afforded methyl 2-*O*-(2,3,4,6-tetra-*O*-methyl-α-D-glucosyl)-3,4-di-*O*-methyl-D-xyloside (III). Acid hydrolysis of III gave crystalline 2,3,4,6-tetra-*O*-methyl-D-glucose and 3,4-di-*O*-methyl-D-xylose, the latter identified as the crystalline lactone (*11*). The high positive rotation ($[\alpha]_D$ + 95° in water) of I indicates that the biose linkage is of the α type. Further proof of the identity of 2-*O*-(4-*O*-methyl-α-D-glucosyluronic acid)-D-xylose (I) was provided by the fact that acetylation of the methyl ester methyl glycoside of this monomethylaldobiouronic acid as described previously (*16, 21*) provided the crystalline methyl 2-*O*-[methyl-(2,3-di-*O*-acetyl-4-*O*-methyl-α-D-glucosid) uronate]-3,4,-di-*O*-acetyl-α-D-xyloside (IV), which proved to be identical with a specimen prepared from white birch wood (*24*).

The crystalline *O*-4-*O*-methyl-α-D-glucosyluronic acid-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-β-D-xylose (Vb), isolated by controlled hydrolysis of the hemicellulose, was found to have the same properties ($[\alpha]_D$ + 52° in water, melting point 178°, OCH_3 7.6, and equivalent weight 540) as those displayed by the mono-*O*-methylaldotriouronic acid isolated from western hemlock (*8*) and jute fiber hemicellulose (*23*). Reduction of methyl [methyl-*O*-(4-*O*-methyl-α-D-glucosyl) uronate-(1→2)-*O*-β-D-xylopyranosyl-(1→4)]-D-xyloside (VI) derived from *O*-4-*O*-methyl-α-D-glucosyluronic acid-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-β-D-xylose (Vb) by the action of methanolic hydrogen chloride at room temperature, followed by hydrolysis, yielded 1 molar proportion of 4-*O*-methyl-D-glucose and 2 molar proportions of D-xylose, thus proving that compound V was a mono-*O*-methylaldotriouronic acid. When the methyl [methyl-*O*-(4-*O*-methyl-α-D-glucosyl) uronate-(1→2)-*O*-β-D-xylopyranosyl-(1→4)]-D-xyloside (VI) was successively subjected to methylation, lithium aluminum hydride reduction, and remethylation, the fully methylated trisaccharide methyl *O*-[2,3,4,6-tetra-*O*-methyl-α-D-glucosyl-(1→2)-*O*-3,4-di-*O*-methyl-β-D-xylosyl-(1→4)]-2,3-di-*O*-methyl-D-xyloside (VII) was produced. Hydrolysis of this trisaccharide (VII) gave 2,3,4,6-tetra-*O*-methyl-D-glucose, 3,4-di-*O*-methyl-, and 2,3,di-*O*-methyl-D-xylose. These results indicated that the mono-*O*-methylaldotriouronic acid was either 4-*O*-methyl-D-G_pA-(1→2)-D-Xyl_p(1→4)-D-Xyl_p or 4-*O*-methyl-D-G_pA-(1→4)-D-Xyl_p-(1→2)-D-Xylp. (Gp = glucosyluronic acid; Xylp = xylopyranosyl).

A decision between these two formulations for the mono-*O*-methylaldotriouronic acid (V) was based on the observation that methanolysis of compound V and subsequent acetylation gave rise to methyl 2-*O*-[methyl-(2,3-di-*O*-acetyl-4-*O*-methyl-α-D-glucosyl)uronate]-3,4-di-*O*-acetyl-α-D-xyloside (IV), thus establishing that the 4-*O*-methyl-D-glucuronic acid residue was linked to C_2 of one of the D-xylose residues. This D-xylose residue clearly corresponds to that which gave rise to 3,4-di-*O*-methyl-D-xylose when methyl *O*-[2,3,4,6-tetra-*O*-methyl-α-D-glucosyl-(1→2)-*O*-3,4-di-*O*-methyl-β-D-xylosyl(1→4)]-2,3-di-*O*-methyl-D-xyloside (VII) was hydrolyzed. The 2,3-di-*O*-methyl-D-xylose obtained from VII must therefore be derived from a 1→4 linked reducing D-xylose residue located in a terminal position.

On the grounds that most xylan polysaccharides contain β-linkages, a deduction reached from the fact that xylans display a relatively high negative rotation, it has been inferred (*8, 22, 23*) that

the 1→4 linkage between the two xylose units of the mono-*O*-methylaldotriouronic acid (V) is of the β- type and consequently would have the structure 4-*O*-methyl-α-D-GpA-(1→2)-β-D-Xylp-(1→4)-D-Xyl. Using Hudson's optical superposition rules and specific rotations (in water) of +150° for methyl 4-*O*-methyl-α-D-glucosiduronamide (*21*, *26*), −50° for methyl 4-*O*-methyl-β-D-glucosiduronamide (*21*, *26*), +154° for methyl α-D-xylopyranoside, −66° for methyl β-D-xylopyranoside, +94° for α-D-xylose, and −30° for β-D-xylose (*3*), specific rotations were calculated for the four mono-*O*-methylaldotriuronic acids. The calculated specific rotations of +134° and +105° for 4-*O*-methyl-α-D-GpA-(1→2)-α-D-Xylp-(1→4)-α-D-Xyl and 4-*O*-methyl-α-D-GpA-(1→2)-α-D-Xylp-(1→4)-β-D-Xyl, respectively, are much higher than that determined experimentally (+45°). The calculated specific rotations of +65° and +33°, respectively, for 4-*O*-methyl-α-D-GpA-(1→2)-β-D-Xylp-(1→4)-α-D-Xyl (Va) and 4-*O*-methyl-α-D-GpA-(1→2)-β-D-Xylp-(1→4)-β-D-Xyl (Vb) are in fair agreement with the observed value of +45°, indicating that the linkage between the two D-xylose residues is of the β- type. The fact that the mono-*O*-methylaldotriouronic acid displays an upward mutarotation (+45°→+52°) requires that the terminal reducing D-xylose unit exist in the β- form. From these data the mono-*O*-methylaldotriouronic acid is tentatively designated *O*-4-*O*-methyl-α-D-glucosyl-uronic acid-(1→2)-*O*-β-D-xylopyranosyl-(1→4)-β-D-xylose (Vb) (*23*).

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RICE AMYLOSES

Molecular Weights of Crystalline Amyloses from Certain Rice Varieties

HSIU YING TSAI, ALLEN T. PHILLIPS, and VIRGINIA R. WILLIAMS

Department of Agricultural Chemistry and Biochemistry, Louisiana State University, Baton Rouge, La.

ALTHOUGH the composition of rice with regard to its major constituents has been studied extensively, the processing characteristics of any variety cannot be predicted on the basis of present compositional information. Certain helpful generalizations can be made regarding amylose-amylopectin ratios and probable cooking quality, but exceptions can always be found (*19*). Consideration of grain type is also useful, but not infallible. Fundamental research is needed in all phases of rice composition to facilitate a more rational interpretation of existing information.

Because the principal chemical constituent of rice is starch, any differences in the chemical and physical properties innately pertaining to the different types of starch present should be reflected in the properties of the rice itself. For this reason, it was desirable to study the physical properties of the amyloses of rices of widely different processing characteristics. The present report describes a study of the crystallization and molecular weight determination of amyloses from four distinctly different varieties: Rexoro, Zenith, Century Patna, and Caloro.

Experimental

Samples of Zenith, Century Patna, and Rexoro rices were obtained from Crowley,