Recent Investigations on Wheat Flour Pentosans[1]

H. NEUKOM, L. PROVIDOLI, H. GREMLI, and **P. A. HUI,** Department of Agricultural Chemistry, Swiss Federal Institute of Technology, Zurich, Switzerland

ABSTRACT

A purified preparation of arabinosidase has been made and shown to liberate arabinose from an arabinoxylan from wheat flour. The removal of arabinose is followed by the precipitation of an insoluble xylan. The degradation of a glycoprotein from wheat flour with Pronase has been investigated. Two polysaccharides with different sugar compositions are formed, indicating that they are linked by a polypeptide bridge. Possible carbohydrate protein linkages are discussed. A caffeic acid ester of starch has been prepared as a model compound for the study of the oxidative gelation of flour pentosans.

Wheat flour pentosans (1,2) have two unique properties which distinguish them from the common hemicelluloses of the plant cell wall: 1) about 20–25% of the total pentosans in a white wheat flour are readily dispersible in water, forming highly viscous solutions; 2) aqueous flour extracts or solutions of purified flour pentosans form solid gels upon addition of minute amounts of oxidizing agents (e.g., H_2O_2, $NaClO_2$, $NaIO_4$). These properties must be due to special structural features not found in other pentosans or related gums.

In attempts further to elucidate the structure of the pentosans, the effect of a purified hemicellulase on pentosans has been studied. Also, experiments have been carried out both on the nature of the protein-carbohydrate linkage in the glycoprotein fractions and on the synthesis of model compounds for studies on the oxidative gelation.

MATERIALS AND METHODS

Preparation of a Purified Hemicellulase (Alpha-L-Arabinofuranosidase)

An aqueous solution of Pectinol R-10 (Rohm & Haas, Philadelphia) was treated with 70% methanol and the resulting precipitate dialyzed and lyophilized. Fractionation of the enzymes was performed on a calcium phosphate column by the method of Tiselius (3). The freeze-dried powder was applied to the column in 0.02M phosphate buffer (pH 5.7). Elution with 0.05M buffer of the same pH gave a fraction showing a high alpha-L-arabinofuranosidase activity measured with phenyl-alpha-L-arabinofuranoside (4) and sugar-beet araban as substrates. Only weak activities of cellulase and amylase could be observed. This fraction was entirely free of xylanase, pectinase, and polygalacturonase activities. (The latter enzymes were eluted only after the buffer concentration was increased to 0.4M.) The arabinosidase showed a pH optimum of 3.8 to 4.0 in McIlvaine's buffer.

Action of Alpha-L-Arabinofuranosidase on Wheat Flour Arabinoxylan

Equal parts of a 1% solution of freeze-dried arabinoxylan (fraction 1 of the DEAE-cellulose chromatography of flour pentosans (5)) and alpha-L-arabinofuranosidase in McIlvaine's buffer (pH 4) were mixed. After incuba-

tion overnight at room temperature a strong turbidity could be observed. Centrifugation at 17,000 r.p.m. yielded a white precipitate which was washed in water and centrifuged once more. A portion of the precipitate (5 mg.) was hydrolyzed in $0.5N$ H_2SO_4 for 16 hr. at 107°C. Paper-chromatography (ethylacetate pyridine H_2O, 8:2:1) showed xylose and only a trace of arabinose.

Preparation of Glycoprotein 2

Wheat flour was mixed with twice its weight of water and extracted according to Kündig et al. (5). The lyophilized flour extracts were treated with crystalline alpha-amylase in $0.02M$ phosphate buffer, pH 7.2 (5); the enzyme was inactivated by trichloroacetic acid (TCA) and removed in a Spinco ultracentrifuge. The supernatant was dialyzed for 24 hr. and concentrated in vacuo. The starch-free pentosan preparation was fractionated on DEAE-cellulose (Brown Co., Berlin, N. H.) to give five fractions (5). Fraction 2, eluted with $0.01M$ sodium borate, is designated as glycoprotein 2. This fraction is composed of xylose, arabinose, and galactose and contains 13–17% protein (5,6). The amino acid composition in the hydrolysate ($6N$ HCl, 22 hr.) was determined with a Beckman Spectrochrom analyzer.

Degradation of Glycoprotein 2 with Pronase

A portion of glycoprotein 2 (200 mg.) was dissolved in 20 ml. of water and 20 ml. of $0.2M$ phosphate buffer, pH 7.3. After addition of 10 mg. Pronase (a protease from Streptomyces griseus, California Corp. for Biochemical Research, Los Angeles), the reaction mixture was incubated at 40°C. for 24 hr. The precipitated enzyme was centrifuged and the supernatant diluted with ethanol. The precipitated polysaccharides were filtered, washed with ethanol, dissolved in a small amount of water, dialyzed, and lyophilized. The Pronase treatment was repeated twice more. The fractions precipitated by alcohol were hydrolyzed and chromatographed. Xylose and arabinose only (no galactose) were found, in addition to some residual protein. The amino acid composition of this protein was determined as described. The alcohol-soluble fraction consisted of amino acids and peptides as shown by TLC (7). Dialysis of this fraction gave a high-molecular-weight residue which upon hydrolysis gave arabinose and galactose (ratio 3:7) and some residual protein, but contained no xylose (7).

Preparation of an Alanine-Xylose Ester

A portion (10 g., $0.05M$) of methyl-3,5-O-isopropylidene-xylofuranoside and 11.15 g. ($0.05M$) of N-carbobenzoxy-DL-alanine were dissolved in 100 ml. of dry pyridine to which 12.5 g. of N,N'-dicyclohexylcarbodiimide in 30 ml. of pyridine was added (7). The mixture was reacted at room temperature for 48 hr. Pyridine was removed in vacuo and the residue extracted with ether. The ether layer was extracted with water and 2% sodium bicarbonate solution. After addition of benzene to the ether layer, the solution was evaporated to dryness, giving a yellowish oil of methyl-3,5-O-isopropylidene-2-O-(N-carbobenzoxy-DL-alanyl)-xylofuranoside. A portion (8.38 g., $0.02M$) of this compound was dissolved in 55 ml. of acetone, 4.82 ml. of concentrated HCl in 68 ml. of water was added, and the mixture was refluxed at 45°C. for 3½ hr. The acetone was evaporated and to the residue 120 ml. of water

and 80 ml. of ether were added. The water layer was extracted with ether, neutralized with silver carbonate, filtered, and extracted with butanol. After removal of the butanol, the residue was dissolved in ethanol and treated with charcoal. Ethanol was evaporated *in vacuo*, yielding a yellowish oil of methyl-2-O-(N-carbobenzoxy-DL-alanyl)-xylopyranoside. A 1.3-g. portion of methyl-2-O-(N-carbobenzoxy-DL-alanyl)-xylopyranoside was dissolved in 30 ml. of MeOH (75%), and 0.26 g. of oxalic acid and 300 mg. of palladium catalyst were added. After hydrogenation for 2 hr. the catalyst was centrifuged off. Addition of ether to the supernatant precipitated an oil which could be separated by centrifugation, dissolved in a small amount of methanol, and treated with ether. The resulting precipitate was dried *in vacuo* over P_2O_5, giving pure 2-O-DL-alanyl-methyl-xyloside-oxalate as an amorphous white powder.

The stability of the ester linkage was tested at various pH values at room temperature. After 8 hr. at pH 1 and 12 hr. at pH 3, the ester was completely hydrolyzed into alanine and methyl xyloside (7).

Preparation of a Caffeic Acid Ester of Starch

The carbomethoxy caffeic acid ester of starch was prepared by reaction of equimolar amounts of starch and the acid chloride (8). Soluble starch was dissolved in dimethylformamide (DMF) and a small amount of pyridine. To this a solution of carbomethoxy caffeic acid chloride in DMF was added dropwise. The reaction mixture was held at 75°C. for 36 hr. and then precipitated by addition to 70% acetone, filtered off, and thoroughly washed with acetone. The resulting product was dried *in vacuo* at 50°C. The protecting carbomethoxy groups were split off by shaking the ester in a mixture of pyridine and 1N ammonia at room temperature for 4 hr. according to Daniels *et al.* (8). The infrared spectra showed the presence of ester bonds (absorption at 1,760 cm.$^{-1}$). The ester obtained was water-insoluble.

Quantitative Determination of Ferulic and Caffeic Acid

Ferulic acid in wheat flour pentosans and caffeic acid in the corresponding starch ester were determined by measuring the ultraviolet absorption of ether extracts at 326 mμ after alkaline saponification of the esters (9). With the use of a calibration curve of the two acids, it is possible to measure very small amounts of ferulic as well as caffeic acid with an experimental error of ±5%.

RESULTS AND DISCUSSION

Starch-free pentosan preparations can be obtained from flour extracts by deproteinization, treatment with crystalline alpha-amylase, and precipitation in alcohol (5). These preparations are primarily composed of the pentose sugars xylose and arabinose but in addition always contain 10–15% galactose and small amounts of proteins (about 8%) which cannot be removed without concurrent loss of pentosan material. It was shown by fractionation on DEAE-cellulose columns (5) that these pentosan preparations are heterogeneous. Five fractions could be obtained which differed in composition. The first and principal fraction was a pure arabinoxylan free of protein and galactose; it corresponds to the pentosan studied by Perlin (10,11) and Mont-

gomery and Smith (12). However, this fraction does not gel upon addition of oxidizing agents. The other four fractions all contained galactose and proteins in addition to pentose sugars and can be designated as glycoproteins. Of these, fraction 2 is the principal and most interesting fraction because it is sensitive to oxidizing agents (gelation) (13). Some new observations on the structure and behavior of these first two fractions will be described.

It is known from the work of Perlin *et al.* (14,15) that fraction 1, the pure arabinoxylan, consists of a straight celluloselike xylan chain to which residues of single arabinofuranose units are attached. The latter are responsible for the water solubility, and their removal by mild acid treatment (to avoid degradation of the xylan chain) has been shown already by Perlin (11) to cause formation of an insoluble xylan. A more selective enzymatic removal of the arabinose residues has now been achieved with the use of an enzyme fraction obtained from Pectinol R-10 by chromatography on calcium phosphate gel. This enzyme hydrolyzed alpha-L-arabinofuranosides and sugar-beet araban but was devoid of xylanase activity and was an almost pure alpha-L-arabinofuranosidase. Incubation of arabinoxylan with this enzyme caused formation of a white precipitate which consisted of a xylan containing only
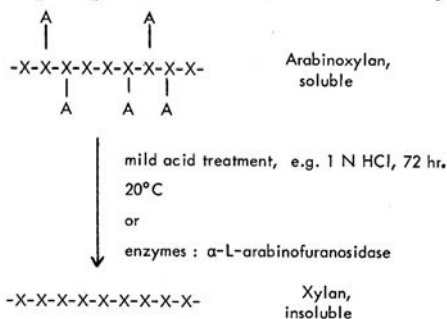

Fig. 1. Transformation of a soluble arabinoxylan into an insoluble xylan.

traces of arabinose (Fig. 1). This observation confirms that the arabinose residues occur in the furanose form and are linked alpha-glycosidically to the xylan chain (14). It remains to be shown whether this mechanism is operative in the formation of the plant cell wall, the soluble arabinoxylans being a sort of precursor for the insoluble xylans (11). The alpha-L-arabinofuranosidase will also be of value for future studies on the structure of the more complex pentosan fractions.

Turning now to the more complicated fraction 2, it has been stated already that it contains galactose and protein (about 13–17%) in addition to xylose and arabinose and is the fraction sensitive to oxidizing agents. The carbohydrate composition (5,6) is remarkably similar to that of the endosperm cell walls of wheat found by Wolf *et al.* (16).

The amino acid composition of the protein part of glycoprotein 2 is shown in Table I. The second column shows the amino acids still present in the pentosan after exhaustive degradation with Pronase, an active bacterial protease. Particularly noteworthy is the high content of alanine, which is greatly increased after Pronase treatment.

TABLE I
AMINO ACID COMPOSITION OF GLYCOPROTEIN 2 BEFORE AND AFTER DEGRADATION
WITH PRONASE[a]

AMINO ACID	BEFORE TREATMENT	AFTER TREATMENT	AMINO ACID	BEFORE TREATMENT	AFTER TREATMENT
	g./100 g. protein	g./100 g. protein		g./100 g. protein	g./100 g. protein
Lysine	6.50	3.65	Alanine	10.70	23.60
Histidine	1.35	1.89	Cysteine	7.60	.....
Arginine	5.60	5.55	Valine	4.50	4.80
Aspartic acid	9.10	9.70	Methionine	1.35	2.10
Threonine	5.90	7.10	Isoleucine	3.30	3.80
Serine	7.20	7.20	Leucine	4.80	5.45
Glutamic acid	18.00	16.10	Tyrosine	3.70	3.80
Proline	5.10	5.50	Phenylalanine	3.50	3.95
Glycine	4.00	4.10			

[a] A protease from *Streptomyces griseus,* California Corp. for Biochemical Research, Los Angeles.

So far, two special structural features have been established in this glycoprotein. 1) It contains very small amounts of ferulic acid, apparently esterified with the xylan chain (9). A ferulic acid content of about 0.5% has been found in this fraction; on the basis of the xylan content only of this fraction, this percentage increases to 1.5%. After oxidation of the pentosans this acid can no longer be detected, and it must be involved somehow in the gelation mechanism. It should be mentioned at this stage that ferulic and the closely related caffeic acid have also been found as esters with fatty alcohols in the lipids of oatmeal, where they seem to be the principal antioxidant material (8). Further, Wolfrom and Patin (17) suspected very recently that some of the coffee-bean hemicelluloses are also esterified with these acids. 2) When glycoprotein 2 is treated with pure proteolytic enzymes like Pronase, the glycoprotein is split into two high-molecular-weight fractions, one being an alcohol-insoluble arabinoxylan containing some residual protein but, remarkably, no galactose, the other an alcohol-soluble but not dialyzable arabinogalactan containing no xylose but some residual protein (7,18). This arabinogalactan was composed of 70% of galactose and 30% of arabinose. A similar arabinogalactan was obtained by mild acid treatment of glycoprotein 2 (6). These results show that the galactose is not linked directly to the xylan chain but rather occurs as an arabinogalactan which very likely is connected via a polypeptide bridge to the arabinose of the arabinoxylan. The known facts about the structure of this complicated glycoprotein are summarized in the structural scheme of Fig. 2.

The most important problems which remain to be solved are the nature of the carbohydrate-protein linkages and the reactions leading to the oxidative gelation of the pentosans. Figure 2 shows that there are probably at least three different types of carbohydrate-protein linkages (1–3, Fig. 2) between xylose, arabinose, and possible galactose units and amino acid residues of the protein part. The efforts are concentrated upon enzymatic breakdown of this glycoprotein, leading eventually to a compound composed of a sugar and amino acid residue which incorporates the carbohydrate protein linkage.
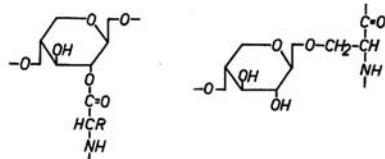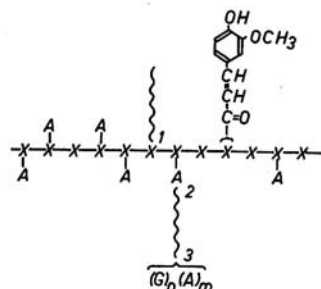
Fig. 2 (left). Hypothetical structure of glycoprotein 2 from wheat flour. Waved vertical line indicates polypeptide chain; X, β-D-xylopyranose units; A, α-L-arabinofuranose units; G, galactose units; 1,2,3, possible carbohydrate protein linkages.

Fig. 3 (right). Two possible linkages between xylose and amino acid units. Left, ester linkage; right, xylosylserine linkage.

Despite many efforts, it has not been possible so far to resolve this difficult task (7). The most likely linkage between protein and xylan chains is an ester bond between carboxyl groups of amino acid residues and the secondary hydroxyls of the xylose units (Fig. 3, left). Such a linkage has been postulated in barley albumin, which contains carbohydrate material and is also a glycoprotein (19). Hochstrasser (19) has claimed the isolation of an alanine-xylose ester from barley albumin after digestion ($5N$ formic acid, refluxed for 12 hr.). We have synthesized such an ester and shown that it is completely hydrolyzed under these conditions (7). It is therefore impossible to isolate these compounds from other than enzymatic digests of glycoproteins. Other possible linkages could involve the reducing end group of the xylan chain which could form an O-glycosidic linkage with the hydroxyl groups of serine or threonine (Fig. 3, right). It is very interesting that such a linkage has indeed been found recently in animal mucoproteins (20). The presence of xylose units in animal tissue is particularly noteworthy.
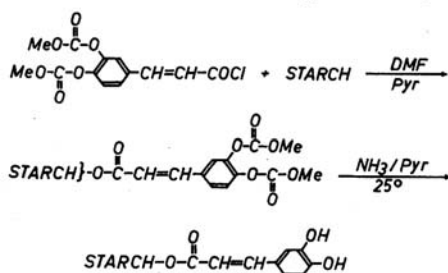

Fig. 4. Preparation of the caffeic acid ester of starch.

Since the nature of the gelation reaction cannot be deduced before the exact structure of glycoprotein 2 is known, an attempt has been made to duplicate this gelation through the use of model compounds containing phenolic groups which are readily oxidized. The starch ester of caffeic acid has been prepared, therefore, by reaction of dicarbomethoxy caffeic acid chloride with starch in dimethylformamide and pyridine. Removal of the protective carbomethoxy groups gave the free caffeic acid ester (Fig. 4). An

ester with a degree of substitution of about 0.3 was obtained by reaction of equimolar amounts of starch and the acid chloride. However, no gelation has been observed so far. These interesting starch esters might also have other useful properties; for example, they could serve as special antioxidants or protein and metal ion precipitants (by complex formation). It is hoped, therefore, that the study of wheat flour pentosans will also uncover new ways to form gels and novel derivatives of polysaccharides.

Literature Cited

1. BECHTEL, W. G., GEDDES, W. F., and GILLES, K. A. Carbohydrates. *In* Wheat: Chemistry and technology, I. Hlynka, ed., chap. 7. Monograph Series, Vol. III. American Association of Cereal Chemists: St. Paul, Minn. (1964).
2. POMERANZ, Y. Cereal gums. Qualitas Plant. Materiae Veget. 8: 158–194 (1961).
3. TISELIUS, A., HJERTEN, S., and LEVIN, Ö. Protein chromatography on calcium phosphate columns. Arch. Biochem. Biophys. 65: 132–155 (1956).
4. BÖRJESON, H., JERKEMAN, P., and LINDBERG, B. Phenylglycofuranosides. Synthesis of phenyl-β-D-xylofuranoside and phenyl-α-L-arabinofuranoside. Acta Chem. Scand. 17: 1705–1708 (1953).
5. KÜNDIG, W., NEUKOM, H., and DEUEL, H. Chromatographische Fraktionierung von wasserlöslichen Weizenmehlpentosanen an DEAE-cellulose. Helv. Chim. Acta 44: 823–829 (1961).
6. KÜNDIG, W. Untersuchungen über die Zusammensetzung und Eigenschaften der Schleimstoffe des Weizenmehls. Thesis, Federal Inst. Technol., Zurich, 1962.
7. PROVIDOLI, L. Untersuchungen über wasserlösliche Glycoproteine in Getreidemehlen. Thesis, Federal Inst. Technol., Zurich, 1965.
8. DANIELS, D. G. H., KING, H. G. C., and MARTIN, H. F. Antioxidants in oats: esters of phenolic acids. J. Sci. Food Agr. 14: 385–390 (1963).
9. FAUSCH, H., KÜNDIG, W., and NEUKOM, H. Ferulic acid as a component of a glycoprotein from wheat flour. Nature 199: 287 (1963).
10. PERLIN, A. S. Isolation and composition of the soluble pentosans of wheat flours. Cereal Chem. 28: 370–381 (1951).
11. PERLIN, A. S. Structure of the soluble pentosans of wheat flours. Cereal Chem. 28: 382–393 (1951).
12. MONTGOMERY, R., and SMITH, F. Constitution of a water-soluble hemicellulose of the endosperm of wheat. J. Am. Chem. Soc. 77: 3325–3328 (1955).
13. KÜNDIG, W., NEUKOM, H., and DEUEL, H. Über die Gelierung wässriger Lösungen von Weizenmehlpentosanen durch Oxydationsmittel. Helv. Chim. Acta 44: 969–976 (1961).
14. EWALD, C. M., and PERLIN, A. S. The arrangement of branching in an arabinoxylan from wheat flour. Can. J. Chem. 37: 1254-1259 (1959).
15. GOLDSCHMID, H. R., and PERLIN, A. S. Interbranch sequences in the wheat arabino-xylan. Can. J. Chem. 41: 2272–2277 (1963).
16. WOLF, M. J., SECKINGER, H. L., ROSEWALL, E. C., MACMASTERS, MAJEL M., and RIST, C. E. Studies of water-insoluble hemicelluloses of the endosperm cell walls in relation to milling quality of seven Pacific Northwest wheat varieties. Cereal Chem. 29: 399–406 (1963).
17. WOLFROM, M. L., and PATIN, D. L. Carbohydrates of the coffee bean, an arabinogalactan. J. Org. Chem. 30: 4060–4062 (1965).
18. KÜNDIG, W., and NEUKOM, H. Untersuchungen über Getreideschleimstoffe. VI. Abbau eines Glycoproteins mit proteolytischen Enzymen. Helv. Chim. Acta 46: 1423–1426 (1963).
19. HOCHSTRASSER, K. Über die Bindungsart von Kohlehydrat in Gerstenalbumin. Isolierung von 4-D-Alanyl-L-xylopyranose als Brückenglied. Z. Physiol. Chem. 234: 250–253 (1961).
20. GREGORY, J. D., LAURENT, T. C., and RODEN, L. Enzymatic degradation of chondromucoprotein. J. Biol. Chem. 239: 3312-3320 (1964).