Use of Calcofluor in Analysis of Oat Beta-D-Glucan[1]

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ABSTRACT

Addition of the fluorescent whitening agent Calcofluor to solutions of partially purified oat (1→3) (1→4)-β-D-glucan gave precipitates that, on acid hydrolysis and high performance liquid chromatography, showed glucose as the only monosaccharide present. Solutions obtained by extraction of oat flour with carbonate buffer at pH 10 also gave precipitates in the presence of Calcofluor. The β-D-glucan determined by analysis of the material precipitated by Calcofluor was in good agreement with the values obtained from the difference between total glucan and α-glucan.

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The endosperm and aleurone cell walls of oats and barley are rich in (1→3) (1→4)-β-D-glucan (Bacic and Stone 1981, Wood et al 1983). This polysaccharide can adversely affect the performance of barley for malting and brewing (Bourne et al 1976) and has been implicated as the factor responsible for the low metabolizable energy of barley as poultry feed (Novacek and Petersen 1967). Oat β-glucan may have therapeutic value as a soluble dietary "fiber" (Chen et al 1981), and its rheological properties make it a potentially useful food hydrocolloid (Wood et al 1978). A reliable and rapid analysis of this polysaccharide is, therefore, essential.

One approach has been to measure total glucan colorimetrically (Wood et al 1977) or by acid hydrolysis (Fleming and Kawakami 1977), and to subtract starch glucose, which is measured enzymically. The difference is then considered β-glucan. The disadvantage of this approach is that complete conversion of starch to glucose may be difficult, and the vigorous extraction conditions necessary for complete solubilization of β-glucan concomitantly increase the starch content of the sample to be analyzed, and hence increase the error. These methods assume extractable glucan to be either starch or the (1→3) (1→4)-β-D-glucan rather than, for example, xyloglucan.

An alternative approach has been to use enzymes specific for hydrolysis of β-glucan. Anderson et al (1978) used a highly purified and characterized preparation from *Bacillus subtilis* that was specific for cleavage of (1→3) (1→4)-β-D-glucan. Oligosaccharides released were extracted and converted to glucose, which was then measured.

More convenient assays, in which β-glucanase preparations were used to convert β-glucan to glucose, have been described (Prentice et al 1980, Martin and Bamforth 1981), but great caution must be taken to ensure both complete inactivation of amyloglucosidases in the crude enzyme mixture and consistent conversion of β-glucan to glucose (Gill et al 1982).

The two essential requirements for (1→3) (1→4)-β-D-glucan analysis are completeness of extraction and distinction between

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starch, or α-glucans, and β-glucan. The approach that we describe to the latter problem does not depend on enzymes because these may not be readily available in the required purity.

The method is dependent upon specific precipitation of (1→3) (1→4)-β-D-glucan by dyes such as Calcofluor and Congo red, essentially as described previously (Wood and Fulcher 1978, Wood 1980). Values for (1→3) (1→4)-β-D-glucan in oats obtained by dye precipitation (3.45%) compared well with conventional analysis (3.58%) (Wood 1980), but a somewhat laborious gravimetric procedure requiring removal of bound dye was used. Workers at the Carlsberg laboratories have also described a method using Calcofluor, but their technique was dependent upon correlation with other methods (Jensen and Aastrup 1981). We describe here an approach in which the dye-β-glucan complex is hydrolyzed by an acid and the released glucose determined by high performance liquid chromatography (HPLC).

To test the method, a selection of samples of oat "gum" isolated by precipitation with 2-propanol was used essentially as described previously (Wood et al 1977, 1978). Calcofluor (0.25 ml, 20 mg/ml) (American Cyanamid Co., Bound Brook, NJ) was added to aliquots (3 ml) of gum (0.1%; w/v) dissolved in sodium carbonate/bicarbonate buffer, pH 10, ionic strength 0.2. Precipitates isolated by centrifugation (12,000 × *g*; 20 min), were hydrolyzed in 0.5*M* trifluoroacetic acid (2 ml) (TFA) at 125°C for 1 hr. TFA was removed in a stream of air at 60°C, the sample dissolved in filtered, glass-distilled water (0.5 ml), passed through a small column of Dowex 50W × 8 H^+ form (0.5 ml-resin bed), and Dowex 1 × 2 Cl^- form (0.5 ml-resin bed) and sample plus aqueous washings diluted to 2.0 ml. The samples were then filtered through a Millipore filter (0.45 μm) and analyzed by HPLC using aqueous elution from a Bio-Rad HPX-85 heavy metal carbohydrate column with refractive-index monitoring. Only a single peak with the retention time of glucose was detected. The total glucan content in the original gum was similarly determined using TFA hydrolysis and HPLC. Starch content was determined by a modification of the method of Batey (1982).[2] Values obtained by both methods for 18 samples of gum are shown in Table I. Because both methods contain a random component, and because neither can be considered to provide a standard or absolute value, the use of ordinary regression procedures is invalid. The procedure used instead was that given by Williams (1959), in which the slope of the observed relationship is compared to the line that would be obtained if the methods agreed precisely (ie, a line passing through the origin with slope 1). The F-ratio obtained (0.069) showed that the agreement with the theoretical relationship was good. Despite this, our experience suggest that slightly lower values are generally obtained with the Calcofluor technique, but the precision of the methods was not sufficient to demonstrate statistical significance.

The β-glucan extracted by alkali from oat flours was also determined. Duplicate samples of flour (0.9 g) were extracted by stirring with sodium carbonate/bicarbonate buffer (ionic strength 0.2, pH 10.0) (9.0 ml) for 0.5 hr at 45°C. The extract was centrifuged (12,000 × *g*, 20 min) and the supernatant diluted to 10 ml with buffer. The residue was re-extracted with buffer (8.0 ml) twice, and each supernatant diluted to 10 ml to give consecutive extracts numbered 1, 2, and 3. One aliquot (5 ml) of each extract was adjusted to pH 4.5 with HCl, heated at 100°C for 5 min to inactivate enzymes, cooled and centrifuged (12,000 × *g*, 20 min), and the supernatant transferred to dialysis bags containing a drop of chloroform. After 20 hr of dialysis against tap water and 2 hr against distilled water, the solutions were diluted to 25 ml (less for some extracts), and aliquots analyzed for total glucan as described by Wood et al (1977), and for starch as described earlier.

Aliquots (2.5–5 ml) of the remaining supernatant of each alkaline extract were also analyzed by Calcofluor precipitation, essentially as described for the oat gum samples, but the extracts 1, which were viscous, were diluted with the carbonate buffer (1:1) before addition of the Calcofluor (0.25 ml; 20 mg/ml). In one experiment, five replicate aliquots were removed from an extract, treated with Calcofluor, and the precipitates analyzed by HPLC. Again, only a single peak with the retention time of glucose was detected on HPLC.

The results with a number of different oat flours are shown in Table II. Because the flour samples were prepared by different grinding and milling techniques, the results do not represent whole kernel analyses. Similar to the results of Table I, both methods include a random component. Statistical analysis by the same procedure (Williams 1954) after logit transformation[3] of the figures to allow for the clustering at the low end of the percentage scale

[2]The enzymes used (α-amylase from Calbiochem, catalogue number 171568, lot 103424; and amyloglucosidase from BDH catalogue number 39075, lot 1611020) did not release glucose from (1→3) (1→4)-β-D-glucans (J. Weisz and P. J. Wood, *unpublished*).

[3]$y = \log (x/100\text{-}x)$ where x is measured percentage = logit transformation.

TABLE I
Comparison of Assays for (1→3) (1→4)-β-D-Glucan Content of Oat Gum

Sample	Total Glucan[a] (TFA[b] hydrolysate)	Starch[a]	β-D-Glucan[a] By Difference[c]	β-D-Glucan[a] Calcofluor Method
1	70.8	15.9	54.9	50.4
2	73.5	5.0	68.5	61.0
3	74.8	6.3	68.5	70.9
4	69.7	3.5	66.2	64.9
5	65.4	4.1	61.3	67.5
6	66.9	6.0	60.9	60.0
7	69.1	2.8	66.3	66.8
8	69.3	11.6	57.7	59.1
9	70.6	3.7	66.9	63.2
10	65.1	10.4	54.7	57.0
11	68.5	2.9	65.6	65.5
12	65.2	8.7	56.5	56.6
13	70.1	3.9	66.2	60.5
14	68.4	6.1	62.3	63.2
15	69.5	2.9	66.5	59.8
16	60.0	2.6	57.4	59.5
17	65.6	9.4	56.2	62.0
18	67.3	3.2	64.1	63.0

[a]Percent, as is basis.
[b]Trifluoroacetic acid.
[c]Total glucan - starch.

TABLE II
Comparison of Two Estimates of (1→3) (1→4)-β-D-Glucan Extracted From Oats by Carbonate Buffer (pH 10) at 45°C

Sample	Extract	Percent β-D-Glucan[a] By Difference[b]	Percent β-D-Glucan[a] Calcofluor Method
1	1	3.06	2.62
	2	0.84	0.85
	3	0.15	0.11
	Total	4.05	3.58
2	1	3.01	3.39
	2	1.14	0.87
	3	0.16	0.14
	Total	4.31	4.40
3	1	2.85	2.41
	2	0.81	0.65
	3	0.04	0.06
	Total	3.70	3.12
4	1	2.41	2.40
	2	0.75	0.81
	3	0.01	0.04
	Total	3.17	3.25
5	1	2.70	2.80
	2	1.57	1.39
	3	0.67	0.48
	Total	5.22	4.88

[a]Average of duplicates expressed on a dry weight basis.
[b]Total glucan - starch.

showed that the two methods were in good agreement (F-ratio = 0.0107). The replicated sample (n = 5) showed a mean of 2.86 mg of β-glucan in each precipitate, with a coefficient of variation of ±4.70% and a range of 2.66–3.07 mg. Again, in all the samples, essentially only a single peak with the retention time of an authentic sample of glucose was detected on HPLC. Any method in which Calcofluor does not interfere with the measurements might be used to analyze the glucan precipitate, which usually contains approximately equal proportions by weight of dye and β-glucan.

It is not our intention to deal here with the problem of incomplete extraction. However, use of elevated temperature to increase extraction, as described by Fleming and Kawakami (1977), Wood et al (1978), and Prentice et al (1980), can be successfully applied despite increased starch contamination. For example, in flour sample 5 (Table II), a starch content of 5% was determined when extraction was at 45°C. When extraction was for 2 hr at 80°C despite a higher starch content in the extract (17%), both Calcofluor and the difference method showed a 6.34% β-glucan value under these latter conditions.

These results confirm our previous reports that Calcofluor may be used to quantitatively precipitate (1→3) (1→4)-β-D-glucan in extracts from oats and demonstrates a rapid analytical application. Efforts to extend this approach to other cereals, using improved extraction procedures, are in progress.

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