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ORIGINAL PAPER

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Determination of β-glucan content of cereals with an amperometric glucose electrode

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Abstract A rapid method for determination of the soluble and total amount of β-glucan was investigated. β-Glucan was extracted from the samples with the modified extraction method and then incubated with lichenase and β-glucosidase. The optimum incubation parameters were determined by using pure β-glucan solutions as follows: lichenase concentration, 0.04 international units (IU) ml^{-1}; β-glucosidase concentration, 0.125 IU ml^{-1}; reaction time, 20 min; reaction temperature, 50 °C; reaction acidity, pH 6. After enzymatic incubation, free D-glucose produced by the action of enzymes on soluble and total β-glucans was measured with an amperometric glucose electrode. The linearity of the β-glucan assay was determined as 8.0% (w/w) under the considered enzymatic reaction condition. In the last part of the study, soluble and total β-glucan contents of certain samples were determined by both the improved method and the AOAC Official Method. A high correlation (R^2: 0.992) between the results of these two methods was observed.

Keywords β-Glucan · Cereals · Lichenase · β-Glucosidase · Glucose · Amperometric enzyme electrode

Introduction

The (1→3),(1→4)-β-glucans are homopolysaccharides which occur at different levels in variants of cereals and cereal products. These homopolysaccharides occur in large quantities particularly in the endosperm cell wall of the barley and bran of oats [1, 2, 3]. β-Glucans have been studied in particular for the brewing industry and for their properties as dietary fiber. In the brewing industry, they are responsible for poor wort separation, difficulties in beer filtration and formation of undesirable precipitates [4]. Due to their having these properties, several methods have been developed for the determination of β-glucans in cereals and cereal products. [1, 2, 3, 4, 5].

Problems occurring in these methods often include incomplete extraction or undesirable hydrolysis of β-glucans during extraction [5, 6]. One of the most important points is avoiding the hydrolysis of β-glucans from endogenous enzymes. Alkali or acid solutions are used for the extraction of β-glucans if their concentrations are high enough to inactivate the endogenous enzymes [2]. Besides these, hot water, whose temperature is above the inactivation temperature of enzymes, is used for the extraction of β-glucans [5].

After extraction, β-glucan contents of samples are determined with viscosimetric, enzymatic and colorimetric methods [3]. The most popular one is the enzymatic method developed by McCleary and Glennie-Holmes [3, 7]. The principal of the method is depolymerization of β-glucans into oligosaccharides by lichenase and hydrolysis of these oligosaccharides into glucose by β-glucosidase. Then glucose is oxidized to gluconic acid and hydrogen peroxide with glucose oxidase and hydrogen peroxide is measured in the presence of peroxidase, spectrophotometrically. By using the stoichiometry of the enzymatic reaction sequence, the β-glucan contents of samples can be calculated. The reactions involved are:

$$\beta\text{–Glucans} \xrightarrow{\text{Lichenase}} \text{Oligosaccharides} \quad (1)$$

$$\text{Oligosaccharides} \xrightarrow{\beta\text{–Glucosidase}} \beta\text{–D–Glucose} \quad (2)$$

$$\beta\text{–D–Glucose} + O_2 \xrightarrow{\text{Glucoseoxidase}} H_2O_2 + \text{Gluconic acid} \quad (3)$$

$$2H_2O_2 + \text{p–hydroxybenzoic acid} + 4\text{–aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + 4\,H_2O \quad (4)$$

The objective of our study was to develop a rapid and sensitive method for determination of soluble and total

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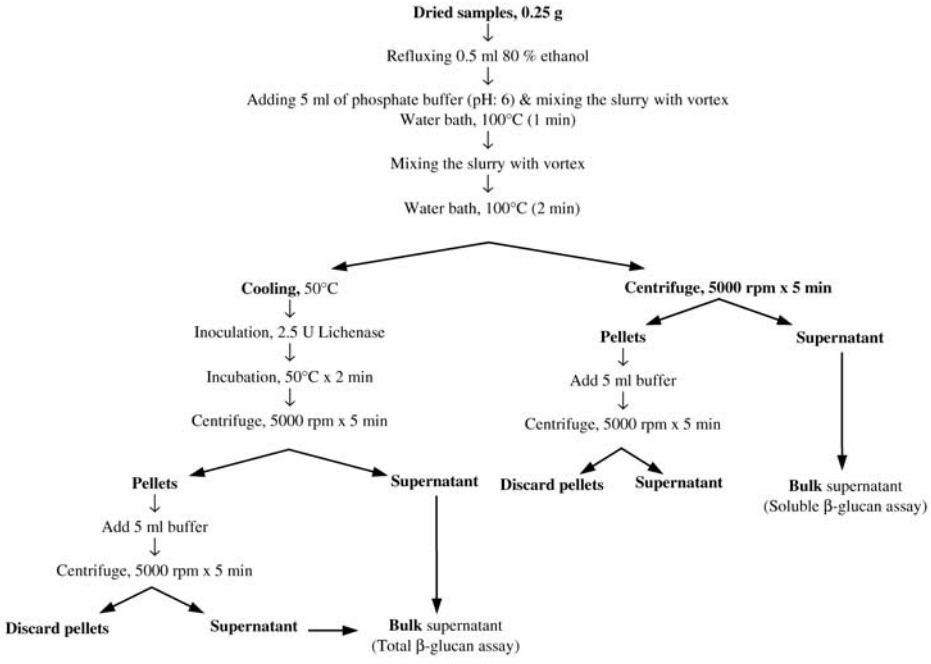

Fig. 1 A flow chart showing soluble and total β-glucan extraction methods

β-glucans in cereals. β-Glucans were obtained from samples with the modified extraction method. In order to reduce analysis time and increase sensitivity, firstly the enzymatic hydrolysis of β-glucans was tested to investigate optimum analytical procedure, and then glucose obtained from β-glucans was measured with an amperometric glucose electrode. After that soluble and total β-glucan contents of cereal samples were determined by this improved method. The method of McCleary-Mugford [8], which is recognized as AOAC International Reference Method, was also used to measure the β-glucan contents of the samples. The correlation between the results of AOAC Official Reference Method and the improved method in this study was determined for testing the utility of the new method.

Materials and methods

Materials. Lichenase (EC 3.2.1.73 from *Bacillus subtilis*: 118 international units (IU) mg^{-1}), β-glucosidase (EC 3.2.1.21 from *Aspergillus niger*: 53 IU mg^{-1}) and a β-glucans assay kit (K-BGLU) were purchased from Megazyme (Ireland). Glucose oxidase (EC 1.1.3.4 from *Aspergillus niger*: 219 IU mg^{-1}), glutaraldehyde (25% aqueous solution), β-glucans (extracted from barley), D-glucose and buffer components were supplied by Sigma (UK). Polycarbonate membranes (0.03 μm pore size) were obtained from Poretics (USA). Wheat and barley samples were supplied by the Turkish Ministry of Agriculture Plant Research Institute (Ankara, Turkey).

Preparation of amperometric glucose electrode. The glucose electrode was prepared by contacting the glucose recognition layer on the amperometric hydrogen peroxide electrode probe. A hydrogen peroxide electrode produced in our laboratory was used. The electrode consists of a Pt working electrode and a Ag/AgCl reference electrode. The Pt electrode was polarized at 650 mV (vs. Ag/AgCl) for hydrogen peroxide detection; the meter was linked to the data acquisition system of the computer. Glucose electrodes of different performance parameters were investigated in our previous studies [9, 10, 11]. A highly sensitive glucose electrode would seem to be more convenient for determination and quantification of β-glucans, due to the nature of the enzymatic reaction sequences. In this study, a sandwich type glucose recognition layer was prepared. Glucose oxidase was immobilized between two polycarbonate membranes by using glutaraldehyde as a cross-linking agent. After preparation, the performance parameters of the glucose electrode were determined on the bases of linearity, sensitivity, and response time. More details on the preparation of glucose electrode have been given in our previous publication [9].

Sample preparation and extraction. The extraction method was obtained by modification of the method developed by Carr et al. [5]. In this new approach, total β-glucans were not extracted directly. Firstly, they were hydrolyzed into oligosaccharides with lichenase in the sample sludge, and then the oligosaccharides, which are water-soluble, were extracted with boiling water. So, an extract with which to determine total β-glucans had been prepared.

Barley and wheat flours were used as the test samples. Samples were dried at 105 °C for 2 h. The 0.25 g dried samples were wetted with 0.5 ml of 80% ethanol (to homogenize the sludge and to reduce the boiling temperature of water) and 5 ml of phosphate buffer (pH 6) was added to the slurry. The slurry was placed in a water bath (100 °C) for 3 min while mixing with a vortex. In the soluble β-glucan extraction, heat-treated slurry was centrifuged at 5,000 rpm for 5 min and then the supernatant was removed. The pellet was mixed with 5 ml buffer to extract the probable soluble β-glucan residues, and the mixture was centrifuged again. The supernatants taken from each extraction step were collected and the pellet was discharged. In total β-glucan extraction, heat-treated samples were cooled to inoculation temperature (50 °C) and total β-glucan was hydrolyzed into oligosaccharides with 2.5 IU of lichenase in 2 min. These water-soluble oligosaccharides were extracted with the same procedure described for the soluble β-glucan assay. A flow chart of extraction of soluble and total β-glucans is given in Fig. 1.

Optimization of enzymatic reaction conditions. After the extraction processes, β-glucan had to be hydrolyzed into β-D-glucose by lichenase and β-glucosidase. For that reason, conditions of the enzymatic reactions were optimized on the bases of enzyme concentration, reaction time, temperature and pH. An optimization of li-

chenase concentration was achieved by increasing enzyme concentration from 0.000 to 0.200 IU ml^{-1} (other parameters were kept constant at: β-glucosidase concentration, 0.06 IU ml^{-1}; temperature, 60 °C, pH 6.0; incubation time 30 min). A further test was performed to optimize β-glucosidase concentration at constant lichenase concentration, incubation temperature, pH and time (0.125 IU ml^{-1}, 60 °C, pH 6.0 and 30 min, respectively). Different β-glucosidase concentrations in the range 0.000–0.100 IU ml^{-1} were tested. A test was also performed to estimate the optimum incubation time. It was tested between 0 and 30 min (0.04 IU ml^{-1} β-glucosidase and 0.125 IU ml^{-1} lichenase, pH 6.0, at 60 °C). The reaction temperature was also tested in the range 10–60 °C at constant reaction parameters (0.04 IU ml^{-1} β-glucosidase and 0.125 IU ml^{-1} lichenase, pH 6.0, for 20 min). Finally, the reaction acidity was tested between the parameters of pH 4.0 and pH 8.0 (0.04 IU ml^{-1} β-glucosidase and 0.125 IU ml^{-1} lichenase, 50 °C, for 20 min). In this part of the study, pure β-glucan solutions, prepared from 0.5 g l^{-1} β-glucan stock solution, were used as control and calibration.

In order to determine the maximum measurable β-glucan concentration in the enzymatic reaction conditions considered, ten model samples whose β-glucan concentrations were known (in the range 0.0–10.0%) were prepared from stock solution.

Soluble and total β-glucan assay. The extracts obtained from soluble and total β-glucan extraction procedures and model samples were incubated with 0.125 IU ml^{-1} lichenase, 0.04 IU ml^{-1} β-glucosidase, at 50 °C and pH 6.0 for 20 min, separately. In order to homogenize the reaction media, it was mixed continuously. After incubation, 500 µl of product was used for glucose assay.

The glucose measurements were carried out in 5 ml phosphate buffer in which a glucose electrode was immersed. An incubated sample (500 µl) was transferred into the measuring volume and its electrode response was measured. Also, a blank containing sample incubated without lichenase and β-glucosidase was prepared to avoid possible errors that might be caused by free glucose from the media or starch hydrolysis, and its electrode response was determined.

β-glucan assay of samples with the McCleary method. The soluble and total β-glucan contents of the samples were also determined using the McCleary Method, according to AOAC Official Method 995.16 [8].

All measurements were determined in triplicate and average values and averages of absolute deviations were calculated. Finally, the accuracy of the method improved in this study for β-glucan assay was tested by comparing its results with the AOAC Official Method's results.

Results and discussion

Performance of the glucose electrode

An amperometric glucose electrode was prepared with a sandwich-type recognition layer. The performance parameters of the electrode were determined as linearity, 2.5 g l^{-1}; sensitivity, 63.7 nA l g^{-1} D-glucose and response time, 32 s. The electrode which was produced for that particular measurement showed lower linearity but much higher sensitivity when compared with our previous studies [9, 10, 11].

Optimization of enzymatic reaction conditions

Increasing lichenase concentrations caused an increase in electrode response up to 0.125 IU ml^{-1}, after which the difference in electrode response was no longer significant. Similar behavior was observed up to 0.04 IU ml^{-1} β-glucosidase in the presence of a fixed amount of lichenase. These concentrations of enzymes were selected as optimum.

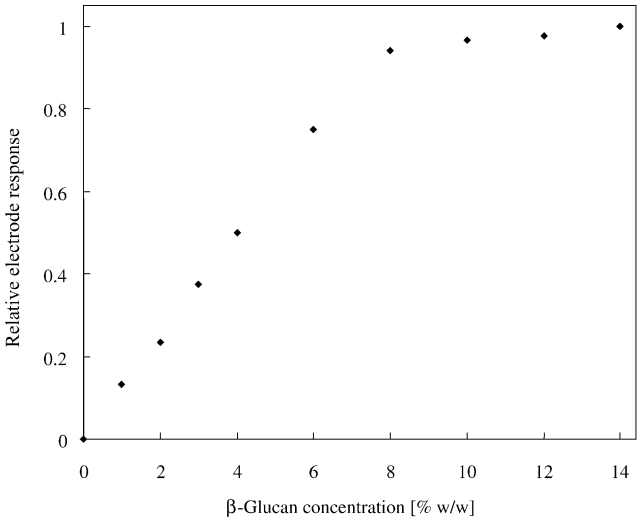

Fig. 2 The linearity graph of the improved β-glucan assay

In order to save time, the reaction periods for lichenase and β-glucosidase were also optimized. The reaction time for lichenase was only 1 min and that for β-glucosidase was 20 min, which meant that lichenase was reaching the plateau value 20 times faster than β-glucosidase. So, we decided to incubate these enzymes together for 20 min.

In the range 10–50 °C, an increase in temperature resulted in an increase in enzyme activity. Over a temperature of 50 °C, a sharp decrease in activity was seen, which meant that 50 °C was optimum as incubation temperature. The acidity of the media was also tested for incubation. The enzymatic activity increased with an increase in pH from 4 to 6 and then a sharp decrease was observed. So, the optimum pH was determined as pH 6.

Soluble and total β-glucan assays

As seen in Fig. 2, an increase in β-glucan concentration also caused an increase in the response of the glucose electrode up to 8.0% (w/w), linearly. Above this concentration, this linear relationship disappeared, which meant that the upper limit of β-glucan determination for the defined parameters was 8%. This upper limit was reasonable for a β-glucan assay of cereals in which β-glucan content varies within a range of 0.5%–7% [12].

In the final part of the research, soluble and total β-glucan contents of cereal samples were determined. The electrode responses due to the D-glucose produced from soluble and total β-glucans were calculated by subtraction of the blank response from the sample responses. After that, soluble and total β-glucan content of the samples were calculated by using the equation given below.

Table 1 The comparison of soluble and total β-glucan contents of samples obtained from the improved method with amperometric glucose electrode and AOAC Official Method

Samples		Improved method with amperometric glucose electrode		AOAC Official Method (McCleary method)	
		Soluble β-glucan % w/w	Total β-glucan % w/w	Soluble β-glucan % w/w	Total β-glucan % w/w
Wheat Flour	Bezostaya	0.15±0.02	0.20±0.02	0.14±0.01	0.18±0.01
	Gün	0.44±0.03	0.67±0.03	0.41±0.03	0.65±0.04
Barley Flour	Bülbül-dehusked	1.52±0.06	2.23±0.17	1.46±0.09	2.37±0.21
	Bülbül-husked	1.62±0.10	2.51±0.15	1.59±0.08	2.46±0.18

$$G = \frac{\Delta I \times F \times DF}{S \times W} 100 \quad (5)$$

where G was the β-glucan concentration (the weight in grams of the β-glucans/the weight in grams of the dried sample as a percentage), ΔI the electrode response in nanoamps, DF the dilution factor×sample volume (0.110), in liters, S the sensitivity in nanoamp liters/gram, W the weight of dried samples in grams, and F the conversion factor from glucose to glucose fragment in β-glucan (162/180), 0.9.

Soluble and total β-glucan contents of samples were also measured using the AOAC International Reference Method [8] and the results are given in Table 1. It is clearly seen that the results of these two methods for different samples were totally matched, with high correlation (R^2=0.992). This high correlation gives us the possibility of using this method as an alternative to AOAC methods with high accuracy. This improved method has some basic advantages. The major one is that it is possible to extract soluble and total β-glucan in a shorter time than with conventional methods. Furthermore, optimum incubation parameters determined on the bases of enzyme concentration, reaction period, temperature and acidity gave us the possibility of reducing the incubation period and analysis cost. In AOAC International Reference Method for determination of β-glucan, the total time for extraction and incubation is 80 min where it is just 25 min with our method. The most important advantage of our method is that we can directly measure the glucose produced from β-glucan by a simple amperometric glucose electrode. It gives us an opportunity to measure glucose in as short a time as 32 s, whereas glucose measurement in the AOAC method needs 20 min incubation period.

In conclusion, it can be said that the method proposed in this study is preferable to the standard method, due to its fast analysis time and relatively low analysis cost. This method also simplifies the operation as well as increasing its speed. We believe that it can be used for routine and efficient measurements of soluble and total β-glucan contents of cereals.

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