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Quantification of β-glucans in Barley – Review
Zjišťování obsahu β-glukanů v ječmeni – review

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The increasing interest in defining the content of β-glucans is related to the development of methods for their determination. Several methods of determination have been published. At present, the most widespread McCleary's enzymatic method is based on the specific hydrolysis of β-glucan by enzymes with β-glucanase activity and the determination of free glucose, which has become the official AACC, AOAC, and ICC method. The second most widely used method is the Flow Injection Analysis (FIA) method based on fluorescence detection of the Calcofluor complex with β-glucans. This method has become the official EBC method. In addition, modifications of the enzymatic method and other methods of determination – chromatographic and spectrometric - are surveyed.

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S nárůstem zájmu o zjištění obsahu β-glukanů souvisí i rozvoj metod jejich stanovení. Publikováno bylo vícero způsobů stanovení. V současné době je nejrozšířenější McClearyho enzymatická metoda založená na specifické hydrolýze β-glukanů enzymy s β-glukanasovou aktivitou a stanovení volné glukosy, která se stala oficiální metodou AACC, AOAC, ICC. Druhou nejvíce používanou je metoda průtokové injekční analýzy (FIA) založená na detekci fluorescence komplexu Calcofluoru s β-glukany. Tato metoda se stala oficiální metodou EBC. Dále jsou v článku zmíněny modifikace enzymové metody a další způsoby stanovení – chromatografické a spektrometrické.

Keywords: barley, β-glucan, enzymatic method, Calcofluor, HPLC
Klíčová slova: ječmen, β-glukany, enzymatická metoda, Calcofluor, HPLC

1 INTRODUCTION

β-glucans are non-starch polysaccharides formed by unbound chains of glucopyranose units linked by glycosidic mixed β-(1,3),(1,4) or β-(1,3),(1,6). They are part of the cell walls of bacteria, fungi, yeasts, algae, lichens ((1,3),(1,6)-β-D-glucans) and higher plants ((1,3),(1,4)-β-D-glucans), in larger quantities are found in the seeds of some cereals (Velíšek, 1999).

In barley, the β-D-glucans content most often ranges between 2–8% (Pérez-Vendrell et al., 1995) and have both malting and nutritional significance (Macháň et al., 2014). The content of β-glucans in malting barley is monitored because β-glucans, together with pentosans (arabinoxylans), increases the viscosity of wort and beer and can cause problems in their separation and filtration (Basařová et al., 2015). Higher β-glucans content is not desirable even for barley intended for monogastric feed (piglets, poultry) that cause digestive problems and reduce nutrient utilization (Prugar et al., 2008). In human nutrition, however, they have a positive meaning, especially because of their bioactive and medicinal properties (Zeković et al., 2005). β-glucans are a component of soluble fibre that has the ability to lower cholesterol, regulate blood sugar, reduce the risk of heart disease and colon cancer and particularly (1,3)-β-D-glucans are sought because of their immunomodulatory effects, nevertheless isolated high molecular weight mixed-linked β-glucans and arabinoxylans from barley show low immunological responses in selected in vitro test systems (Samuelsen et al., 2011). Therefore, in 2006, the Food and Drug Administration (FDA) approved a health claim on the positive effect of β-glucans from barley on cholesterol reduction and risk of heart disease in daily consumption of 3 g of soluble β-glucans. Claims may be applied to foods that contain at least 0.75 g of soluble β-glucans per serving. EFSA (European Food Safety Authority) issued a statement on β-glucans from oats and barley (EFSA, 2009; EFSA, 2011). On the one hand, the claim to the effect of β-glucans on maintaining a normal cholesterol level that can be used for foods containing at least 1 g of β-glucans in one quantified portion and beneficial effect is achieved with a daily intake of 3 g β-glucans. The second claim concerns the beneficial effects of β-glucans oats and barley on blood glucose.

Increasing interest in finding β-glucan content is also related to the development of methods for their determination. Several methods of determination have been published (Hozová et al., 2007), but the choice of an appropriate extraction technique is important, because it may affect the quality, structure, rheological properties, molecular weight, and other functional properties of the extracted β-glucan (Ahmad et al., 2012). At present, McCleary's enzymatic method and flow injection analysis method are the most widely used.

2 β-GLUCAN ASSAY KIT

This method was published in 1985 (McCleary and Glennie-Holmes, 1985) and remains the world standard procedure for measurement of mixed-linkage β-glucan in barley, malt, wort, bear and oats (Cauvain and Young, 2009). McCleary and Codd (1991) simplified a commercially available enzymatic method for the quantitative measurement of (1,3),(1,4)-β-D-glucan to allow analysis of up to 10 grain samples in 70 min or of 100-200 samples by a single operator in a day. These improvements have been achieved with no loss in accuracy or precision and with an increase in reliability. The glucose oxidase/peroxidase reagent has been significantly improved to ensure colour stability for periods of up to 1 h after development. Some problems experienced with the original method have been addressed and resolved, and further experiments to demonstrate the quantitative nature of the assay have been designed and performed.

The Streamlined β-glucan method (Megazyme method) has been successfully evaluated by AOAC International (Method 995.16), AACC (Method 32-23.01) and ICC (Method No. 166, approved 1998). This method determines quantitative (1,3),(1,4)-β-D-glucan (β-D-glucan, mixed-linkage β-D-glucan) and is applicable to cereal grains (e.g. barley, oat, rye), their milling products and cereal based foods containing high level of glucose, after pre-extraction with aqueous ethanol. The method is a rapid procedure for direct, quantitative measurement of (1,3),(1,4)-β-D-glucan (β-D-using highly purified lichenase and β-glucosidase). β-D-Glucan is specifically hydrolysed by lichenase to oligosaccharides, which are then quantitatively cleaved to glucose by β-glucosidase. Glucose is measured using glucose oxidase-peroxidase-buffer mixture (Cauvain and Young, 2009). The absorbance is measured at 510 nm.

$$\beta\text{-D-glucan} + H_2O \xrightarrow{\text{lichenase}} \beta\text{-D-gluco-oligosaccharides}$$

$$\beta\text{-D-gluco-oligosaccharides} \xrightarrow{\beta\text{-glucosidase}} \beta\text{-D-glucose}$$

$$\beta\text{-D-glucose} + H_2O + O_2 \xrightarrow{\text{glukose oxidase}} \text{D-gluconate} + H_2O_2$$

$$2\,H_2O_2 + p\text{-hydroxybenzoic acid} + 4\text{-aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{quinonimine} + 4\,H_2O$$

The growing interest in β-glucans and the dietary recommendations of an exact daily intake will require rapid and accurate quantification methods of β-glucans that can be used routinely by the food industry. Hu and Burton (2008) modified the current approach to a cost-efficient and high-throughput format without compromising the accuracy of the results. Ten barley (Hordeum vulgare L.) genotypes and 21 oat (Avena sativa L.) samples used in the study represented a wide range of β-glucan content levels. Initial steps resemble original protocol. A reduced reaction volume is used in the new protocol to adapt to a 96-well plate format. Two assay plates are

used, one for β-glucosidase reaction, another for GOPOD reaction. The final volume of the well is 160 µl. As a result of format changes, the volume of key components lichenase and β-glucosidase were reduced to 25%, the cost per sample to 22% and labour cost to 25% of the original protocol. The results indicated that the new protocol consistently produced accurate measurements in both barley and oat comparable to the current standard enzymatic procedure.

Motilva et al. (2014) adapted the standard enzymatic procedure to micro-plate format and further application to analyse cereal based samples with a wide range of (1,3),(1,4)-β-D-glucan content (from 0.27-75%). The samples used in this study included two breads (wheat and barley/wheat), barley flours (4% and 8% β-glucans) and two samples of oat bran (28% and 75% β-glucans). 50 mg was selected as the optimal weight for all sample types, incubation with lichenase was also modified. The supernatant obtained after centrifugation was diluted (1:6), because this sample dilution allowed to adjust the volume of GOPOD reagent (210 µl) to the final volume of the well (250 µl). 20 µl of supernatant and 20 µl of β-glucosidase is used, later GOPOD is added into the same well. Results showed no significant differences in the quantification of β-D-glucans in different samples, sensitivity and reproducibility by using the original or the micro-method. The developed method allows the β-glucan quantification (specifically for mixed-linkage (1,3),(1,4)-β-D-glucan) to be conducted rapidly and by an efficient and sensitive micro-method in a wide range of concentrations.

✉ 3 OTHER ENZYMATIC ASSAYS

The method of Åman and Hesselman (1985) involves complete degradation of starch using a thermostable α-amylase and amyloglucosidase, precipitation of buffer-soluble β-glucans with 80% (v/v) ethanol and degradation of soluble and insoluble β-glucans with a β-glucanase preparation from Rhizomucor pusillus. Buffer-soluble polymers were precipitated with 80% ethanol, and mono- and oligosaccharides formed from β-glucans were isolated in the 80% ethanol extract. Isolated sugars were hydrolysed with acid and the content of total mixed-linkage β-glucans was calculated from the glucose content, as determined by the glucose oxidase method. The different steps in the described method and its precision were investigated. Analytical results on some barley cultivars were compared with results obtained with two previously-described methods.

Carr, et al. (1990) developed an improved method for the determination of (1-3),(1-4)-β-D-glucans in cereal-based food products and cereal grains. The method uses refluxing 80% (v/v) ethanol to remove sugars and inactivate enzymes prior to 1-hr extraction with water at 100 °C for soluble β-glucan determination or 16-hr extraction with 1.0 N NaOH for total β-glucan determination. An enzyme preparation from Penicillium funiculosum (cellulase) is used to selectively and quantitatively liberate glucose from β-glucan in the extracts. For several different food products, soluble glucan content ranged from 0.49 to 3.90%, whereas total β-glucan content ranged from 0.58 to 8.86% (dry weight basis). The pre-extraction with refluxing 80% ethanol is not required for the unprocessed cereal grains.

Saulnier et al. (1994) extracted (1,3),(1,4)-linked-β-glucan from dehusked flour of two barley cultivars, Waxiro and Cameo, by sequential treatment with water at 40 °C, water at 90 °C with heat-resistant pancreatic α-amylase and 1 M NaOH at room temperature. Extracted β-glucan was determined enzymatically (Megazyme). The sequential extraction released all the β-glucan. The distribution of β-glucan in the different extracted fractions was different for the two cultivars.

Pérez-Vendrell et al. (1995) developed an HPLC method for the determination of β-glucan in barley. The β-glucan was hydrolysed with lichenase [endo-β-(1,3),(1,4)-D-glucan-4-glucanhydrolase from Bacillus subtilis] to oligosaccharides, which were analysed by reversed-phase HPLC using water as the mobile phase at a flow-rate of 0.7 ml/min. The separation of the oligosasaccharides was performed in a C_{18} stainless-steel column (Spherisor ODS-2) with 5-µm particles in less than 10 min, with a refractive index detection. Pérez-Vendrell et al. (1996) studied the acid extract viscosities and β-glucan contents of ten two- and six-rowed barley cultivars grown at seven locations in three consecutive years in Spain. The viscosities varied from 2.4 to 24.8 centistokes (cSt) and the mean value was 6.4 cSt. The average β-glucan content of barleys determined by HPLC was 3.5% with a range of 1.9–5.5%. Significant differences were found in both β-glucan content and acid extract viscosity between different cultivars, locations and years. The β-glucan contents and viscosities of winter cultivars were higher than those of spring. Environmental factors influenced both parameters. The acid extract viscosities of barleys were correlated negatively with the amount of precipitation ($r = -0.754$; $P < 0.05$). Barleys grown in wet and rainy areas had lower viscosity values.

Johansson et al. (2004) isolated water-soluble and water-insoluble (1,3),(1,4)-β-D-glucans from whole-grain oats and barley and digested with lichenase. The oligosaccharides thus produced were analysed using anion-exchange chromatography with pulse-amperometric detection (HPAEC-PAD). The FT-IR, ^{1}H NMR and solid-state ^{13}C CP-MAS NMR spectra of the β-glucans were also measured. Analyses of the ratio of oligosaccharides with degrees of polymerisation 3 and 4 (DP3:DP4) showed small structural differences between oats and barley and between the water-soluble and water-insoluble β-glucans. The molar masses analysed using the SEC-HPLC method were 500,000 g/mol for the soluble β-glucans of both oats and barley and <200,000 g/mol for the insoluble β-glucans. No differences were found in the FT-IR and NMR spectra.

✉ 4 CALCOFLUOR-FIA

The second most widely used method is Flow Injection Analysis (FIA) based on the detection of fluorescence of the Calcofluor complex with β-glucan. This method became the official EBC (European Brewery Convention) method 3.10.2 (barley), 4.16.2 (malt and malt wort), 8.13.2 (wort).

In the 70s of the last century, the development of the method was inspired by the need for a simple, precise and fast method of determining β-glucans in barley, malt, malt wort and beer when brewing malting barley. Due to problems with wort separation, beer filtration, and chilling turbidity in beer to which β-glucans strongly contributes, it is desirable that their content be low.

Jørgensen (1988) devised an automatic flow injection analysis (FIA) system based on the specific complex formation between Calcofluor and soluble high molecular weight β-glucan. The fluorescence intensity of the complex, in a solution is proportional to the concentration of β-glucan molecules with a molecular weight above 104. For calibration, standards with known purity of β-glucan are required. Four preparations differing in MW distribution and purity gave similar calibration values. Comparison of the Calcofluor-FIA method with the enzymatic method of McCleary and Glennie-Holmes (1985) showed a high correlation between the values obtained by two methods. The water soluble β-glucan fraction of grains from 23 barley varieties was determined by both methods and yielded a linear correlation coefficient of $r = 0.976$ between the two procedures.

Wu et al. (2008) describes the binding of Calcofluor, a fluorescent probe, to oat β-glucan in buffer solutions. The binding equilibrium constant (K), the total number of binding sites per β-glucan molecule (N), and the average binding number of Calcofluor per β-glucan molecule (n) were determined by UV spectroscopic method. The results indicate that the association of Calcofluor and β-glucan is driven by both enthalpy and entropy and that the process involves hydrogen bonding, van der Waals forces, and hydrophobic interaction. Higher buffer concentration and NaCl facilitate the binding of Calcofluor to β-glucan. The adsorption isotherm fits a Langmuir model quite well.

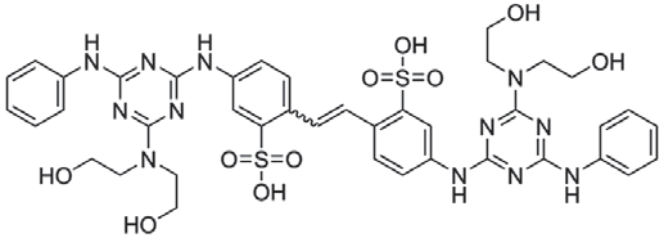

Fig. 1 Schematic structure of Calcofluor molecule {4,4′-bis[4-[bis(2--hydroxyethyl)amino]-6-anilino-1,3,5-triazin-2-yl]amino]stilbene-2,2′--disulphonic acid}.

✉ 5 IR SPECTROSCOPY

Czuchajowska et al. (1992) isolated large and small starch granules from regular amylose (about 25%), high-amylose (44–49%), and high-amylopectin (traces of amylose) barleys. Their near-infra-

red reflectance (NIR) spectra were compared with those of relatively pure (1-3),(1-4)-β-D-glucans from barley and oats and with NIR spectra of ground barleys-naked and covered, regular, high-amylose, and high-amylopectin. The objective was to obtain detailed information on NIR spectra of barleys, isolated starches, and isolated β-D-glucans to serve as a basis for development of a near-infrared spectroscopy method for β-glucan assay in barleys. There was little difference in NIR spectra of hull-less and covered barleys; whole meals of regular, high-amylose, and high-amylopectin barleys showed similar spectra. Small differences were recorded between spectra from high-amylose and regular or high-amylopectin starches. Spectra of starches and whole meals from which the starches were obtained differed widely. Major differences were recorded in three areas over the whole range of 1,600 to 2,400 nm for whole meals, isolated starches, and isolated β-glucans. They included 1) wavelengths 1,702, 1,707, 1,772, and 1,773 nm, principal bands of starches, cellulose, and hemicellulose; 2) 2,060, 2,096, and 2,132 nm, bands associated with the interaction between starch and protein; and, foremost, 3) 2,268, 2,282 and 2,335 nm, bands that typify starch, β-glucans, and cellulose.

Szczodrak et al. (1992) determined total β-glucans in 139 barleys, 84 of which were used for calibration and 55 for validation. Both commercial and varietal samples (from three locations) were used. Equations for prediction of β-glucan content by the best fit of three wavelengths and the single-wavelength (2,264 or 2,348 nm) method (using the step-up program) were developed. The standard error of prediction for both was slightly above 0.6%. The three selected wavelengths were in the 2,260- to 2,380-nm region, previously found to be typical for β-glucans. In light of the highly heterogeneous populations used in this study, the accuracy of predicting the β-glucan contents in the validation samples was affected by the identity of barleys used for developing the prediction equations. Preliminary studies indicated that even for more homogeneous populations, the results may be affected by kernel size, hardness, and protein contents. The above parameters may have to be included in developing equations to predict the β-glucan contents of barleys.

Synytsya et al. (2010) combines quantitative and qualitative evaluation of two spectroscopic methods (FT-IR and FT-NIR) using different statistical methods – hierarchical cluster analysis (HCA), principal component analyses (PCA) and partial least squares (PLS). Using vibrational spectra combined with chemometry, samples of ground grains of barley of different genetic origin can be quickly and non-destructively distinguished. Vibration spectra are very sensitive to differences in chemical composition due to genetic variation of barley. Both methods (FT-IR and FT-NIR) complement each other and combine them to better classify sample fractions of grains according to their composition. For this reason, HCA and PCA of vibration spectra and their derivation can be recommended for the screening of new varieties, breeding lines, genetic resources and other barley samples based on the estimation of the phenotypic impact of certain genetic changes on grain composition including β-glucans. Changes in the content and ratio of the main components of barley grain are strongly reflected in spectroscopic changes that can be detected by chemometric methods. The methodology represents a screening alternative to existing chemical methods and allows for the indicative determination of these parameters using calibration models for FT-IR and FT-NIR.

✉ 6 CONCLUSIONS

Several methods of β-glucan content determination have been published, but the choice of an appropriate extraction technique is also important. The techniques most commonly employed are those based on purified β-glucanase from Bacillus subtilis and fluorometric Calcofluor-FIA methodology. Enzymatic protocols are mostly based on the sequential breakdown of β-glucan to oligosaccharides (lichenase) and glucose (β-glucosidase) and subsequent determination of liberated glucose by the glucose oxidase-peroxidase procedure. Other enzymatic protocols include e.g. enzymes of different origin or determination of oligosaccharides by liquid chromatography. However, several other ways can be used. Calcofluor-FIA method is one of them. It is based on the specific binding of the fluorochrome Calcofluor to β-glucan followed by the fluorometric detection of the complex formed. Completely different way of determination brings NIR/IR spectroscopy and chemometric methods. This indirect way can be used namely for fast and non-destructive screening of large series of similar samples. Although a lot of protocols have been developed it is still desirable to improve its simplicity, throughput and cost-effectiveness without lowering accuracy and precision.

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