Short communication

Determination of fructan exohydrolase activity in the crude extracts of plants

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ABSTRACT

Background: The roots of chicory and the tubers of Jerusalem artichoke are used for the production of inulin. However, a quality of tubers and roots, *i.e.* the content of inulin, monosaccharides and disaccharides, depends on the activity of enzymes implicated in the metabolism of inulin. The knowledge on the changes of activities of inulin synthesizing and degrading enzymes is limited during plant sprouting, growth and dormancy. It happens due to complicated measurements of the product of enzymatic reaction in the presence of crude plant extract. Fructan exohydrolase (β-D-fructan fructohydrolase, FEH, EC 3.2.1.80) is an enzyme responsible for the hydrolysis of fructans in plants. For fructose as the reaction product measurement, a high-performance liquid chromatography is usually used. The aim of the study was to choose a simple and suitable method for FEH activity determination and the measurement of fructose in the presence of plant extracts.
Results: Two chemical methods, *i.e.* copper(II)–neocuproine and 3,5-dinitrosalicylic acid, and the enzymatic one based on the reactions catalyzed by hexokinase, phosphoglucose isomerase and glucose-6-phosphate dehydrogenase were used. Enzymatic method was found to be suitable for FEH activity determination in plant extracts, and on the contrary to chemical methods no interference effects of compounds from crude plant extracts were observed.
Conclusion: Enzymatic method is applicable for the routine analysis and will allow performing the investigations without special equipment on the inulin degrading enzyme in biotechnologically important crops.
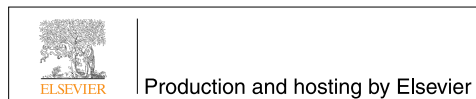
1. Introduction

Fructan exohydrolase (β-D-fructan fructohydrolase, FEH, EC 3.2.1.80) is an enzyme responsible for the hydrolysis of fructans in plants. FEH hydrolyzes fructan molecules at the terminal non-reducing ($2 \rightarrow 1$) and ($2 \rightarrow 6$) linked β-D-fructofuranose residue and release free fructose molecule [1,2]. Fructan is a major reserve carbohydrate found in plants which mainly belong to the *Asteraceae, Campanulaceae, Boraginaceae, Poaceae* and *Liliaceae* families [1]. FEH is found in such plants as *Avena sativa* [3], *Allium cepa* [4], *Hordeum vulgare* [5], *Lolium perenne* [6], *Triticum aestivum* [7], *Smallanthus sonchifolius* [8], *Vernonia herbacea* [9] and *Cynara cardunculus* [10]. Two commercially important plants as *Cichorium intybus* (chicory) and *Helianthus tuberosus* L. (Jerusalem artichoke) also exhibit FEH activity [11,12]. The roots of chicory and the tubers of Jerusalem artichoke are used for the production of inulin. It consists of linear β ($2 \rightarrow 1$) linked fructofuranosyl units terminated by a glucose residue through a sucrose type linkage. Inulin is used in food industry as a soluble dietary fiber and fat or sugar replacement [13,14]. Inulin is also known as a prebiotic and is able to stimulate health-promoting bacterial growth in human colon [15,16]. Moreover, inulin is an important material in bioprocesses and has a high biotechnological potential. Inulin can be used for ethanol and high-fructose syrup production [17]. A quality of tubers, *i.e.* the content of inulin, monosaccharides and disaccharides depends on the activity of enzymes implicated in the metabolism of inulin. There is a lot of information on the variation of those carbohydrates in various plant species. However, the knowledge on the changes of inulin synthesizing and degrading enzyme activities during plant sprouting, growth and dormancy is limited [2,18,19,20,21]. It seems that it happens due to complicated measurements of the product of enzymatic reaction in the presence of crude plant extract. The product of FEH reaction is a reducing monosaccharide fructose. Usually, the content of fructose formed during the reaction is quantified by high-performance liquid chromatography [4,22,23]. However, this method requires special not always available equipment and is time consuming. For fructose quantification, two chemical methods and the enzymatic one were tested.

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2. Materials and methods

2.1. Chemicals

All chemical reagents were of analytical grade. Inulin from *Dahlia* tubers, 3,5-dinitrosalicylic acid, neocuproine hydrochloride hydrate, copper (II) sulfate pentahydrate, potassium–sodium tartrate tetrahydrate, glycine, and sodium carbonate were purchased from Sigma-Aldrich Chemical Co. Glucose and fructose were from Fluka. A kit K-FRUGL 11/05 for the determination of D-fructose and D-glucose was purchased from Megazyme International Ireland Ltd.

2.2. Plant materials

The tubers and leaves of *H. tuberosus* L., leaves of *C. intybus* and tubers of *Dahlia* were purchased in the local market. Three cultivars of *H. tuberosus* L., namely, Rubik, Sauliai and Albik that had been previously described were used for experiments [24]. Tubers were peeled and cut in small pieces using a knife (about 0.3 cm of thickness), then were frozen and homogenized using a Bosch blender. Leaves were frozen, then were cut and homogenized. All homogenized plant materials were stored in plastic bags at -18°C.

2.3. Standard solutions

For constructions of calibration curves, the solutions containing fructose in the concentration range of 0–4 mM and the solutions containing additionally 5 or 20 v/v % of plant extract were prepared. For plant extract preparation, 7.5 g of homogenized plant material in 25 mL of water were kept at 80°C for 15 min.

2.4. Determination of reducing sugars by copper(II)–neocuproine method

The measurement was performed according to the procedure of Dygert et al. [25].

Two solutions were prepared. Solution A was prepared by dissolving 4 g of Na_2CO_3 in 60 mL of distilled water. Then 1.6 g of glycine was dissolved and 0.045 g of $CuSO_4 \times 5H_2O$ was added. Finally, solution A was diluted with distilled water to 100 mL. Solution B was prepared by dissolving 0.12 g of neocuproine hydrochloride hydrate in 100 mL of distilled water. Solution B was stored in the dark. For the measurement, 0.4 mL of the mixture of solution A and solution B (1/1, v/v) was added to 0.03 mL of sample and test tubes were kept in boiling water for 15 min. The reaction mixture was diluted with water to 1.08 mL, and the absorbance was measured at 450 nm.

2.5. Determination of reducing sugars by 3,5-dinitrosalicylic acid (DNS) method

The measurement was performed according to the procedure of Saqib and Whitney [26]. DNS reagent was prepared by adding 1 g of DNS and 30 g of sodium potassium tartaric acid to 80 mL of 0.5 N NaOH. The solution was kept at 45°C for the complete dissolution of reagents and then cooled down to room temperature and diluted with distilled water to 100 mL. The solution was stored for two weeks at 4°C. For the measurement, 0.4 mL of DNS reagent was added to 0.1 mL of sample and test tubes were kept at 95°C for 5 min using Eppendorf Termomixer Comfort. The absorbance was measured at 540 nm.

2.6. Determination of reducing sugars by enzymatic method

A kit K-FRUGL 11/05 (Megazyme) for the determination of D-fructose and D-glucose was used. The procedure was performed accordingly as the manufacturer recommends.

2.7. FEH activity assay

The amount of 4 g of homogenized frozen plant material in 4 mL of 0.1 M Na-acetate buffer, pH 4.5, containing 1 mM phenylmethylsulfonyl fluoride and 5 mM EDTA was sonicated for 3 min at 10°C using a Sonics Vibracell VCX 750. Undissolved material was removed by centrifugation at 15,000 × g for 10 min at 4°C and the supernatant was used for FEH activity determination. A volume of 0.2 mL of supernatant was added to 0.8 mL of 3% inulin solution in 0.1 M Na-acetate buffer, pH 4.5 and incubated for 3 h at 30°C. The reaction was stopped by heating at 95°C for 5 min and liberated fructose was measured by the enzymatic method using a Megazyme kit K-FRUGL 11/05 in accordance with manufacturer procedure. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 µmoL of fructose per 1 h under the reaction conditions. The specific activity was defined as $\mu mol \times h^{-1}$ per g of raw plant material.

3. Results and discussion

3.1. Determination of fructose by copper(II)–neocuproine method

Calibration curves for fructose determination by copper(II)–neocuproine method in the absence and in the presence of 5 or 20 v/v % of various plant extracts are presented in Fig. 1. The method is based on the reduction of Cu(II) to Cu(I) by reducing sugars and the formation of colored Cu(I)–neocuproine complexes [25]. In the absence of plant extract the calibration curve is linear in the concentration range of 0–0.6 mM under procedure conditions presented in Section 2.4 and is described by the following equation: $y = 1.615X$, $R^2 = 0.992$. However, in the presence of extract from *H. tuberosus* L. tubers (Fig. 1a) or leaves (Fig. 1b), *C. intybus* leaves (Fig. 1c) and *Dahlia* tubers (Fig. 1d) an obvious deviation from the linearity is observed. Moreover, when the reaction of reducing sugar determination had been completed the samples were turbid. The turbidity developed probably due to the formation of insoluble Cu ion complexes with some compounds that come from plant extract and interfere with the procedure of the determination of reducing sugar. It is obvious that the copper(II)–neocuproine method is not suitable for an accurate measurement of fructose and the determination of fructan exohydrolase activity in the crude extracts of plants. It should be mentioned that copper(II)–neocuproine method was successfully applied for the determination of reducing sugars in wine [27,28]. There is the possibility to minimize the effect of interfering species by the dilution of sample. This approach was used for polyphenols measuring in wine by copper(II)–neocuproine method [29]. However, this approach cannot be applied to the case of fructan exohydrolase activity determination. Usually, in the crude plant extract the level of enzyme activity is not high and the dilution of that extract can minimize the chance to detect the enzymatic activity.

3.2. Determination of fructose by DNS method

The method is based on the reduction of 3,5-dinitrosalicylic acid to the colorant 3-amino-5-nitro-salicylic acid and the oxidation of the aldehyde group of reducing sugars to the carboxylic acid [26,30]. The method is simple, and the reagents are inexpensive. Thus, the method is widely used for the investigation of polysaccharide bioconversion as well as for the determination of polysaccharide hydrolase activity [31,32]. Moreover, it is an assay recommended by the International Union of Pure and Applied Chemistry [26]. The calibration curve for fructose is linear in the concentration range of 0.6–4.0 mM and is described by the following equation: $y = 0.24X-0.09$, $R^2 = 0.999$ (Fig. 2). It is obvious that DNS method is less sensitive as compared with the copper (II)–neocuproine method mentioned above. The addition of plant extract to the fructose sample drastically influences on the calibration curve for fructose. The interval
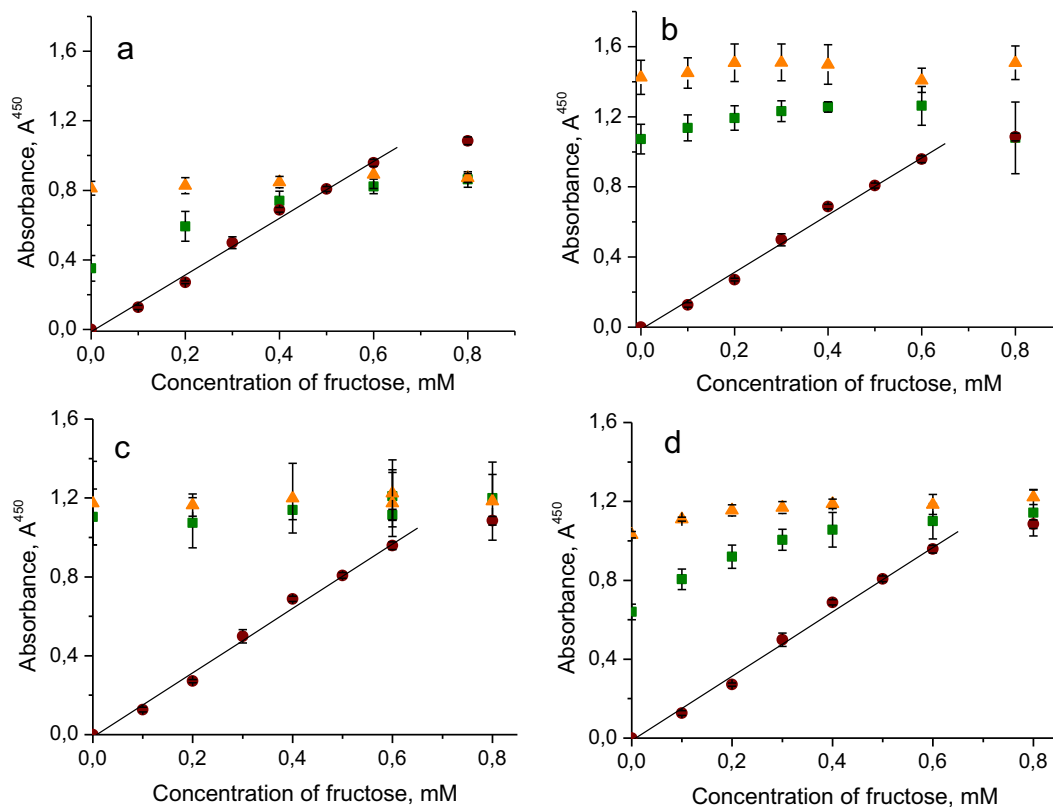
Fig. 1. Calibration curves for fructose determination by copper(II)–neocuproine method in the absence (●) and in the presence of 5 (■) or 20 (▲) v/v % extract from *H. tuberosus* L. (Albik) tubers (a) and leaves (b), *C. intybus* leaves (c) and *Dahlia* tubers (d). Data are presented as mean ± standard deviation of three parallel measurements.

Fig. 2. Calibration curves for fructose determination by DNS method in the absence (●) and in the presence of 5 (■) or 20 (▲) v/v % extract from *H. tuberosus* L. (Albik) tubers (a) and leaves (b), *C. intybus* leaves (c) and *Dahlia* tubers (d). Data are presented as mean ± standard deviation of three parallel measurements.
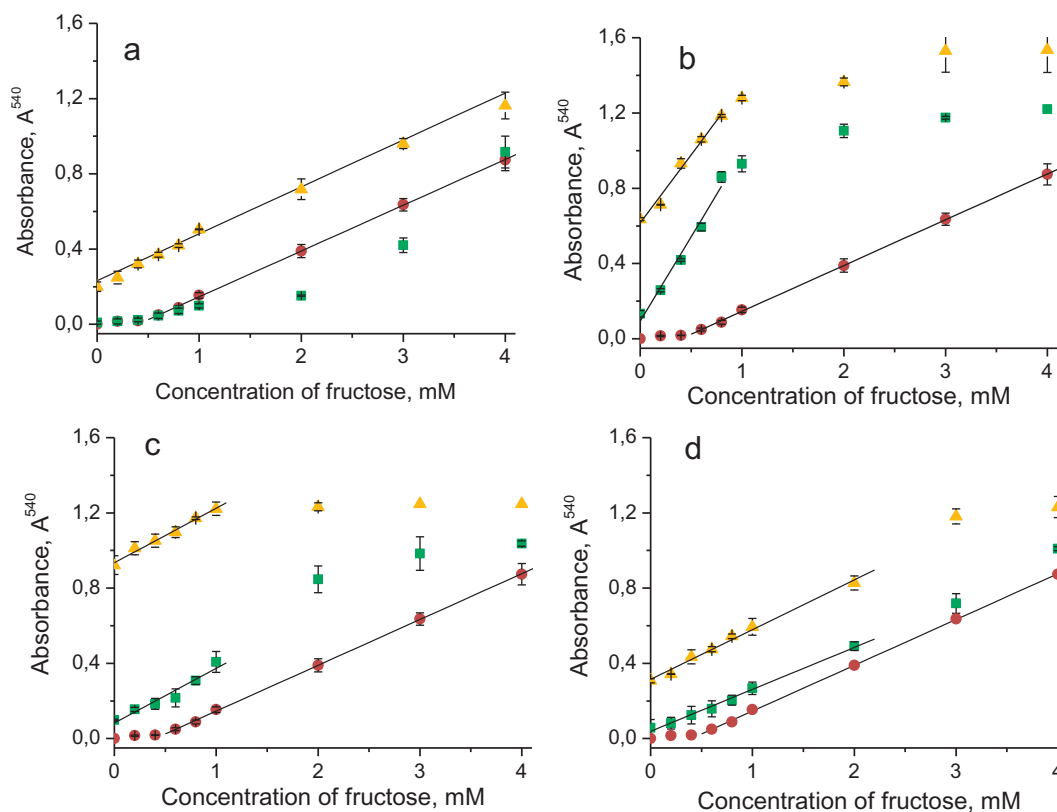
of linearity becomes shorter or the slope of curve changes (Fig. 2). The effect is observed in the case of addition of all plant extracts, but it is different dependently on the plant. Deviations from fructose calibration curve can arise due to the presence of phenolic compounds in plant extracts and the reaction of DNS as a strong oxidator with those compounds. The composition of those compounds and their content differ dependently on the plant. Moreover, it is difficult to compare the data reported in the literature because the composition and the amount of phenolic compounds also depend on the extraction method. Nevertheless, the total amount of phenolic compounds was found to be fourfold higher in the leaves of *H. tuberosus* L. as compared with the tubers [33]. More significant deviation from fructose calibration curve was also observed in the presence of leaf extract (Fig. 2b) as compared with the presence of tuber extract (Fig. 2a). It is difficult to explain a negative interference with DNS method observed in the presence of low concentration of tuber extract equal to 5 v/v % (Fig. 2a). Therefore, the DNS method is not suitable for an accurate fructose measurement and FEH activity determination in the leaves and the tubers of *H. tuberosus* L. as well as in the leaves of *C. intybus* and the tubers of *Dahlia*. There are a lot of publications on the composition and content of phenolic compounds in the leaves of *C. intybus* [34,35,36], but we found no information on these compounds in *Dahlia* tubers. Previously, Xu et al. [37] demonstrated the invalidity of DNS method for glucose measurement in the presence of tea polyphenols.

3.3. Determination of fructose by enzymatic method

The best results were obtained using the enzymatic method. The determination of fructose by Megazyme kit K-FRUGL 11/05 is based on fructose phosphorylation by hexokinase, subsequent conversion of the reaction product fructose-6 phosphate to glucose-6-phosphate by phosphoglucose isomerase and the oxidation of glucose-6-phosphate to gluconate-6-phosphate by $NADP^+$ in the presence of glucose-6-phosphate dehydrogenase. NADPH formed in the reaction is measured by the increase of absorbance at 340 nm. If fructose and glucose are present in the same sample the amount of each sugar can be measured independently or the total amount of both sugars can be determined simultaneously. As can be seen from Fig. 3, calibration curves are linear and practically parallel, and their slope is in the range of 0.22–0.25 independently on the plant extract tested in the experiment. The calibration curves constructed in the presence of plant extract are shifted up along the y-axis because some amount of free fructose and glucose is present in the extracts. If the amount of glucose is subtracted a shift is smaller, and the curves are also parallel to the fructose calibration curve constructed without the addition of plant extract (Fig. 3, open symbols). During the process of FEH activity determination this shift will be eliminated by a blank sample. Therefore, no interference of substances from plant extract was observed, and the enzymatic method is applicable for fructose measurement and FEH activity determination. Using the enzymatic method for fructose measurement, we determined FEH activity in *H. tuberosus* L. leaves and tubers and in *C. intybus* leaves without additional isolation of the enzyme from plant extract (Table 1). It should be mentioned that FEH activity in *Dahlia* tubers was not found. For the experiments, winter tubers were used. It is plausible that at dormancy the level of FEH activity is very low.

4. Conclusions

For FEH activity determination in the crude extracts of plants and the measurement of fructose as the reaction product, we tested two popular chemical methods, *i.e.* copper(II)–neocuproine and 3,5-dinitrosalicylic acid, and the enzymatic one based on the reactions catalyzed by hexokinase, phosphoglucose isomerase and glucose-6-phosphate dehydrogenase. We found that only enzymatic method is suitable
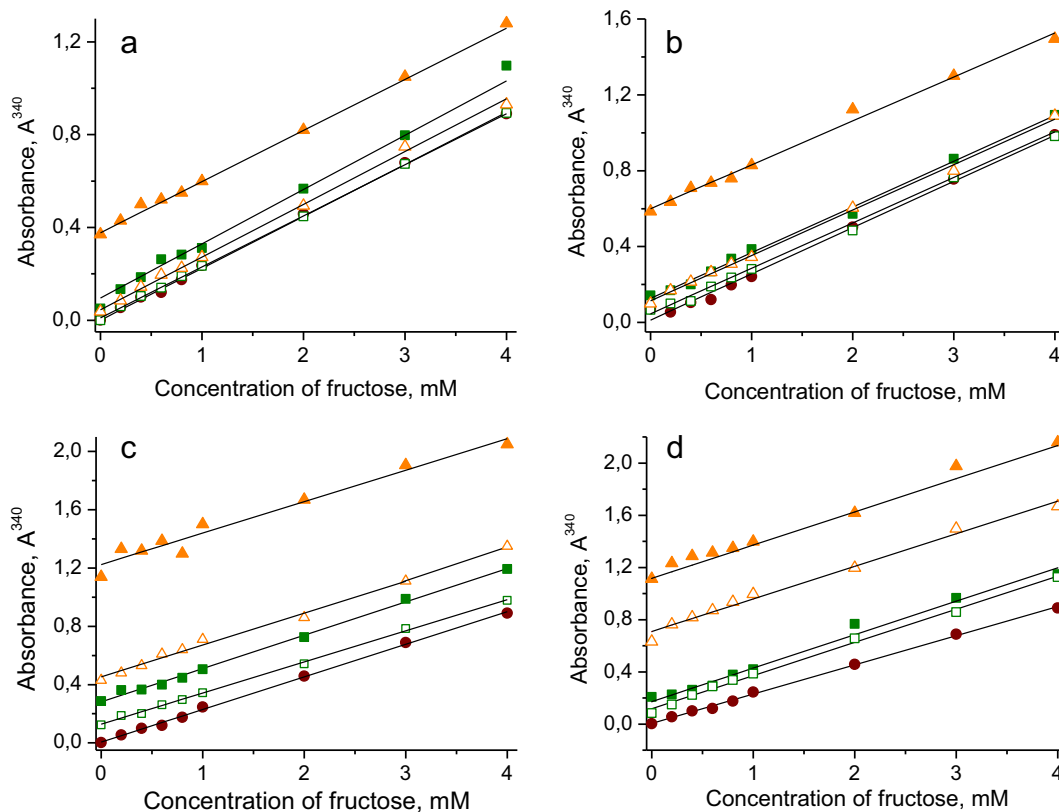

Fig. 3. Calibration curves for fructose determination by enzymatic method in the absence (●) and in the presence of 5 (■) or 20 (▲) v/v % extract from *H. tuberosus* L. (Albik) tubers (a) and leaves (b), *C. intybus* leaves (c) and *Dahlia* tubers (d); □ and △ — the amount of glucose present in the plant extract is subtracted.

Table 1
FEH activity in the crude extracts of various plants.

Name of plant	FEH activity (μmoL $\times$ h^{-1})/g
C. intybus leaves	2.2 $\pm$ 0.5
H. tuberosus L. leaves	0.17 $\pm$ 0.01
H. tuberosus L. tubers of three genetic variants:	
Sauliai	2.6 $\pm$ 0.01
Albik	5.8 $\pm$ 0.02
Rubik	2.5 $\pm$ 0.3

Data are presented as mean $\pm$ standard deviation of three (for leaves) or two (for tubers) parallel measurements.

for FEH activity determination in plant extracts. This method is applicable for routine analysis, and on the contrary to chemical methods was found to be immune to the interference of various compounds from plant extract. The possibility to determine FEH activity in crude extracts, as we exemplified here, will allow establishing the relationship between enzymatic activity and inulin content at various life stages of plants important for inulin production.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this work and do not have a direct financial relation with the commercial identities mentioned in the paper.

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Author contribution

Conceived and designed the experiments: TK, JS; Wrote the paper: JS; Performed the experiments: TK; Analyzed the data: TK, JS.

References

[1] Van den Ende W, De Coninck B, Van Laere A. Plant fructan exohydrolases: A role in signaling and defense? Trends Plant Sci 2004;9:523–8. http://dx.doi.org/10.1016/j.tplants.2004.09.008.

[2] Yildiz S. The metabolism of fructooligosaccharides and fructooligosaccharide-related compounds in plants. Food Rev Int 2011;27:16–50. http://dx.doi.org/10.1080/87559129.2010.518295.

[3] Henson CA, Livingston III DP. Purification and characterization of an oat fructan exohydrolase that preferentially hydrolyzes β-2,6-fructans. Plant Physiol 1996;110:639–44. http://dx.doi.org/10.1104/pp.110.2.639.

[4] Benkeblia N, Ueno K, Onodera S, Shiomi N. Variation of fructooligosaccharides and their metabolizing enzymes in onion bulb (*Allium cepa L.* cv. Tenshin) during long-term storage. J Food Sci 2005;70:S208–14. http://dx.doi.org/10.1111/j.1365-2621.2005.tb07159.x.

[5] Henson CA, Livingston III DP. Characterization of a fructan exohydrolase purified from barley stems that hydrolyzes multiple fructofuranosidic linkages. Plant Physiol Biochem 1998;36:715–20. http://dx.doi.org/10.1016/S0981-9428(98)80021-1.

[6] Lothier J, Lasseur B, Prud'homme MP, Morvan-Bertrand A. Hexokinase-dependent sugar signaling represses fructan exohydrolase activity in *Lolium perenne*. Funct Plant Biol 2010;37:1151–60. http://dx.doi.org/10.1071/FP10086.

[7] Van Den Ende W, Clerens S, Vergauwen R, Van Riet L, Van Laere A, Yoshida M, et al. Fructan1-exohydrolases. β-(2,1)-trimmers during graminan biosynthesis in stems of wheat? Purification, characterization, mass mapping, and cloning of two fructan 1-exohydrolase forms. Plant Physiol 2003;131:621–31. http://dx.doi.org/10.1104/pp.015305.

[8] Narai-Kanayama A, Tokita N, Aso K. Dependence of fructooligosaccharide content on activity of fructooligosaccharide-metabolizing enzymes in yacon (*Smallanthus sonchifolius*) tuberous roots during storage. J Food Sci 2007;72: S381–7. http://dx.doi.org/10.1111/j.1750-3841.2007.00422.x.

[9] Portes MT, Carvalho MAM. Spatial distribution of fructans and fructan metabolizing enzymes in rhizophores of *Vernonia herbacea* (Vell.) Rusby (Asteraceae) in different developmental phases. Plant Sci 2006;170:624–33. http://dx.doi.org/10.1016/j.plantsci.2005.10.017.

[10] Melilli MG, Raccuia SA. Inulin and inulin metabolizing enzyme activities during the growth cycle of wild cardoon. Acta Horticult 2012;942:419–26.

[11] Verhaest M, Lammens W, Le Roy K, De Ranter CJ, Van Laere A, Rabijns A, et al. Insights into the fine architecture of the active site of chicory fructan 1-exohydrolase: 1-Kestose as substrate vs sucrose as inhibitor. New Phytol 2000;174. http://dx.doi.org/10.1111/j.1469-8137.2007.01988.x [790-100].

[12] Marx SP, Nosberger J, Frehner M. Seasonal variation of fructan-β-fructosidase (FEH) activity and characterization of a β-(2–1)-linkage specific FEH from tubers of Jerusalem artichoke (*Helianthus tuberosus*). New Phytol 1997;135: 267–77. http://dx.doi.org/10.1046/j.1469-8137.1997.00641.x.

[13] Barclay T, Ginic-Markovic M, Cooper P, Petrovsky N. Inulin — A versatile polysaccharide with multiple pharmaceutical and food chemical uses. J Excipients Food Chem 2010;1:27–50.

[14] Bosscher D, Frank A. The use of inulin-type fructans in foods to increase satiety, limit energy intake and to control body weight. Agro Food Ind Hi-Tech 2007;18:25–7.

[15] Roberfroid MB, Van Loo JAE, Gibson GR. The bifidogenic nature of chicory inulin and its hydrolysis products. J Nutr 1998;128:11–9.

[16] Gibson GR, Beatty ER, Wang X, Cummings JH. Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin. Gastroenterology 1995;108:975–82. http://dx.doi.org/10.1016/0016-5085(95)90192-2.

[17] Chi ZM, Zhang T, Cao TS, Liu XY, Cui W, Zhao CH. Biotechnological potential of inulin for bioprocesses. Bioresour Technol 2011;102:4295–303. http://dx.doi.org/10.1016/j.biortech.2010.12.086.

[18] Shiomi N, Benkeblia N, Onodera S. The metabolism of the fructooligosaccharides in onion bulbs: A comprehensive review. J Appl Glycosci 2005;52:121–7. http://dx.doi.org/10.5458/jag.52.121.

[19] Melilli MG, Raccuia SA. Influence of shading on flowering induction and inulin metabolism of *Cynara cardunculus* L. Acta Horticult 2013;983:415–20.

[20] Verspreet J, Cimini S, Vergauwen R, Dornez E, Locato V, Le Roy K, et al. Fructan metabolism in developing wheat (*Triticum aestivum* L.) kernels. Plant Cell Physiol 2013;54:2047–57. http://dx.doi.org/10.1093/pcp/pct144.

[21] Fukai K, Ohno S, Goto K, Nanjo F, Hara Y. Seasonal fluctuations in fructan content and related enzyme activities in yacon (*Polymnia sonchifolia*). Soil Sci Plant Nutr 1997; 43:171–7. http://dx.doi.org/10.1080/00380768.1997.10414725.

[22] Morvan A, Challe G, Prud'Homme MP, Le Saos J, Boucaud J. Rise of fructan exohydrolase activity in stubble of *Lolium perenne* after defoliation is decreased by uniconazole, an inhibitor of the biosynthesis of gibberellins. New Phytol 1997;136:81–8. http://dx.doi.org/10.1111/j.1469-8137.1997.tb04733.x.

[23] Imahori Y, Kitamura N, Kobayashi S, Takihara T, Ose K, Ueda Y. Changes in fructooligosaccharide composition and related enzyme activities of burdock root during low-temperature storage. Postharvest Biol Technol 2010;55:15–20. http://dx.doi.org/10.1016/j.postharvbio.2009.08.002.

[24] Krivorotova T, Sereikaite J. Seasonal changes of carbohydrates composition in the tubers of Jerusalem artichoke. Acta Physiol Plant 2014;36:79–83. http://dx.doi.org/10.1007/s11738-013-1388-5.

[25] Dygert S, Li LH, Florida D, Thoma JA. Determination of reducing sugar with improved precision. Anal Biochem 1965;13:367–74. http://dx.doi.org/10.1016/0003-2697(65)90327-1.

[26] Saqib AAN, Whitney PJ. Differential behaviour of the dinitrosalicylic acid (DNS) reagent towards mono- and di-saccharide sugars. Biomass Bioenergy 2011;35: 4748–50. http://dx.doi.org/10.1016/j.biombioe.2011.09.013.

[27] Araujo AN, Lima JLFC, Rangel AOSS, Segundo MA. Sequential injection system for the spectrophotometric determination of reducing sugars in wines. Talanta 2000;52: 59–66. http://dx.doi.org/10.1016/S0039-9140(99)00338-0.

[28] Peris-Tortajada M, Puchades R, Maquieira A. Determination of reducing sugars by the neocuproine method using flow injection analysis. Food Chem 1992;43:65–9. http://dx.doi.org/10.1016/0308-8146(92)90243-U.

[29] Lee G, Rossi MV, Coichev N, Moya HD. The reduction of Cu(II)/neocuproine complexes by some polyphenols: Total polyphenols determination in wine samples. Food Chem 2011;126:679–86. http://dx.doi.org/10.1016/j.foodchem.2010.11.020.

[30] Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 1959;31:426–8. http://dx.doi.org/10.1021/ac60147a030.

[31] Rocha JR, Catana R, Ferreira BS, Cabral JMS, Fernandes P. Design and characterisation of an enzyme system for inulin hydrolysis. Food Chem 2006; 95:77–82. http://dx.doi.org/10.1016/j.foodchem.2004.12.020.

[32] Chen HQ, Chen XM, Li Y, Wang J, Jin ZY, Xu XM, et al. Purification and characterization of exo- and endo-inulinase from *Aspergillus ficuum* JNSP5-06. Food Chem 2009;115: 1206–12. http://dx.doi.org/10.1016/j.foodchem.2009.01.067.

[33] Yuan X, Gao M, Xiao H, Tan C, Du Y. Free radical scavenging activities and bioactive substances of Jerusalem artichoke (*Helianthus tuberosus* L.) leaves. Food Chem 2012; 133:10–4. http://dx.doi.org/10.1016/j.foodchem.2011.09.071.

[34] Heimler D, Isolani L, Vignolini P, Romani A. Polyphenol content and antiradical activity of *Cichorium intybus* L. from biodynamic and conventional farming. Food Chem 2009;114:765–70. http://dx.doi.org/10.1016/j.foodchem.2008.10.010.

[35] Innocenti M, Gallori S, Giaccherini C, Ieri F, Vincieri FF, Mulinacci N. Evaluation of the phenolic content in the aerial parts of different varieties of *Cichorium intybus* L. J Agric Food Chem 2005;53:6497–502. http://dx.doi.org/10.1021/jf050541d.

[36] Mulinacci N, Innocenti M, Gallori S, Romani A, La Marca G, Vincieri FF. Optimization of the chromatographic determination of polyphenols in the aerial parts of *Cichorium intybus* L. Chromatographia 2001;54:455–61. http://dx.doi.org/10.1007/BF02491199.

[37] Xu H, Leng X, Wang M, Zhang G. Glucose measurement in the presence of tea polyphenols. Food Anal Method 2012;5:1027–32. http://dx.doi.org/10.1007/s12161-011-9335-9.