Indirect Enzymatic Assay of D-galactofuranosides of 4-nitrocatechol with an Exoglycosidase from Radish (*Raphanus sativus* L.) Germs

CORINA LOREDANA GUTANU, DUMITRU PETRU IGA*
University of Bucharest, Faculty for Biology, 95 Splaiul Independentei, 050095, Bucharest, Romania

α- And β-L-arabinofuranosides of 4-nitrocatechol were synthesized by a modified Helferich method in the presence of $BF_3 \cdot OBu_2$ as promoter. β- and α-D-galactofuranosides of 4-nitrocatechol were synthesized by Michael method as modified by Mannich, from tetra-O-benzoyl-α-D-galactofuranosyl bromide and 4-nitrocatechol. The latter diastereomeric synthetic glycosides were submitted to oxidation with periodic acid (Malaprade reaction). The new formed aldehyde function was reduced with sodium borohydride and the produced L-arabinofuranosides were chromatographically compared with those directly synthesized and then put in interaction with an enzymatic extract from radish (Raphanus sativus L.) germs. In this way it was indirectly proved that 4-nitrocatechol galactosides syntesized possessed furanosic ring and can be used as substrates for galactofuranosidases.

Keywords: galactofuranoside, 4-nitrocatechol, glycoside, arabinofuranoside, exoglycosidase, NMR spectroscopy

All artificial enzymatic substrates for exoglycosidases – prechromogenic [1-3], chromogenic [4-6] and fluorogenic [7], as well as many natural ones [8], are glycosides of some phenolic compounds. The first synthesis of chromogenic substrates was probably accomplished by [9], the sulfate esters of the three isomers of nitrophenol. In the same period, β-glycosides of 4-nitrophenol with maltose, lactose, cellobiose and gentiobiose were synthesized but the authors did not use these glycosides as enzymatic substrates; they reduced them instead, to 4-aminophenyl glycosides, linked them to a protein by a coupling reaction and used the diazo derivatives for immunological investigations [10]. Next synthesis of chromogenic substrates [4,5] – 4-nitrophenylsulfate, 4-nitrophenylphosphate, 4-nitrophenyl-glucoside, 4-nitrophenyl-galactoside, was accompanied by a demonstration of their use and of advantages concerning chromogenic substrates. Another chromogen was 2-nitrophenol and its glycoside served for isolation and characterization of β-galactosidase [6,11]. Phenolphthalein [12] and p-acetylphenol [13,14] were also used as aglycons for glycosides that proved to be excellent enzymatic substrates.

In this paper, 4-nitrocatechol was as chromogen for the synthesis of α- and β-L-arabinofuranosyl-4-nitrocatechol-1-yl as well as β- and α-D-galactofuranosyl-4-nitrocatechol-1-yl. However, since galactofuranosidases can be found exclusively in pathogenic or extremely pathogenic microorganisms, an indirect enzymatic test was used to prove their usefulness as enzymatic substrates: they were shorten by their non-reducing end by a redox system of reactions and converted to arabinofuranosides. The latter were chromatographically compared with directly synthesized arabinofuranosides and then submitted to the action of arabinofuranosidase from radish (*Raphanus sativus* L.) germs.

Experimental part

Materials and methods

D-Galactose (D-Gal) and 4-nitrophenol were from Sigma. All other reagents used were either from Fluka or from Merck and they were of analytical grade.

All reactions were followed by thin layer chromatography (TLC) analysis: native glycosides were eluted in solvent system (SS) 1, chloroform-methanol-water 60:25:4 (v/v) while acetylated ones were eluted in the SS 2, chloroform–methanol 19:1 (v/v). The plates were visualized by three methods: (a) by exposure to UV light (380 nm); (b) by dipping the plates in a 1 M solution of NaOH in ethanol–water (1:1); (c) by dipping the plates in mostain, followed by heating [15]. The peracylated glycosides were separated by crystallization from ethanol.

The ^1H and ^{13}C NMR spectra of synthetic intermediates and products were acquired in $CDCl_3$ containing TMS. One-dimensional NMR experiments were performed on a Bruker Avance DRX 400 spectrometer using 400 and 100 MHz for the ^1H and ^{13}C frequencies, respectively. The ^1H-^1H correlation spectroscopy (COSY) and ^1H-^{13}C heteronuclear multiple quantum coherence (HMQC) experiments were carried out with an inverse probe.

The molar ratio of glycosides constituents was determined by boiling in sulfuric acid (2 N, 2 h) followed by partition between water and ethyl ether; in the ether phase 4-nitrocatechol was determined and in the water phase the sugar, both colorimetrically [15].

Tetra-O-acetyl-αβ-L-arabinofuranose [16-18]

L-Arabinose (5 g; 33.30 mmol) was dissolved by heating and stirring in 60 mL DMF and to the hot solution a mixture of 40 mL pyridine-acetic anhydride 1/1 (v/v) was added. The solution was then evaporated to dryness by rotavapor and the residue, tetra-O-acetyl-αβ-L-arabinofuranose, repeatedly crystallized from ethanol (0.953 g; 29.97 mmol; 90 %). ^1H and ^{13}C NMR Spectroscopy indicated a reasonable proportion of furanosic isomers:

^1H NMR. ($CDCl_3$; δ ppm; *J* Hz) 6.153 (s, alpha linkage, 1H) (H-1); 4.338 (1H) (H-2); 4.170 (1H) (H-3); 5.315 (1H) (H-4); 4.230 (1H) (H-5a); 4.301 (1H) (H-5b); 1.989-2.118 (Me groups of –OAc linked to sugar).

^{13}C NMR. ($CDCl_3$; δ ppm; *J* Hz) (fig. 1): 99.21 (C-1); 82.25 (C-2); 79.55 (C-3); 74.59 (C-4); 64.45 (C-5); 20.32-20.93 (Me groups of –OAc linked to sugar); 168.94-170.44 (>C=O groups of Ac).

* *email pdiga49@yahoo.com*

4-Nitrocatechol. 4-Nitrocatecholsulfate (2-hydroxy-5-nitrophenylsulfate) has been synthesized as indicated [19]. A solution consisted of 1 L water, KOH (70 g; 1250 mmol) and $K_2S_2O_8$ (70 g; 259.25 mmol) was prepared and to it 4-nitrophenol (30 g; 215.82 mmol) was added. The whole mixture was stirred and heated at 37 °C for 48 h, cooled to room temperature, acidified to *pH* 4 with 4 N solution of H_2SO_4 and extracted two times with ether to remove unreacted 4-nitrophenol. The aqueous solution was then made strongly alkaline by adding 5 N KOH and concentrated by rotavapor to about one third. To this suspension, two volume of acetone were added and the whole mixture stirred for 30 min and filtered. The material on the filter was washed with a solution of acetone-water 2/1 (v/v) and the washings were added to the latter filtrate and the whole was concentrated to dryness by rotavapor. The residue was repeatedly crystallized from water (4.2 g 4-nitrocatechol sulfate · $2H_2O$ as K phenoxide, K salt; 12.1 mmol; 5.6 %). This phenoxide sulfate ester can be preserved for years on dry $CaCl_2$ in a dessicator at room temperature.

About 1 g (2.88 mmol) of 4-nitrocatechol sulfate · $2H_2O$, K phenoxide, K salt, was suspended in 120 mL of 2 N H_2SO_4 solution and the whole was boiled for 2 h. The solution was cooled to room temperature, and extracted five times with chilled ether. The whole etheric solution was washed with small volumes of water, dried on $MgSO_4$, filtered, evaporated to dryness and the residue dried in a vacuum dessicator (0.4243 g 4-nitrocatechol; 2.73 mmol; 95 %). A small amount of 4-nitrocatechol was peracetylated and its ^1H and ^{13}C NMR spectra registered [20,21].

Tri-O-acetyl-αβ-L-arabinofuranosyl-4-nitrocatechol-1-yl-2-acetate [15]

Glycosylation donor, tetra-O-acetyl-αβ-L-arabinofuranose (5 g, 15.72 mmol), and 4-nitrocatechol (1,2-dihydroxy 4-nitrobenzene) (2.43 g, 15.67 mmol) were dissolved in 25 mL of CH_2Cl_2 and 2.53 g (12.8 mmol) of $BF_3 \cdot OBu_2$ was added. The mixture was stirred for 2 days at room temperature and then partitioned 3 times between a saturated solution of sodium bicarbonate and CH_2Cl_2. The solution of the latter was dried over $MgSO_4$, filtered, evaporated to dryness, and acetylated by stirring overnight with an excess of Ac_2O/pyridine 1:2 (v/v). Any acetylation reagents were removed by rotavapor and the residue was crystallized from ethanol, tri-O-acetyl-αβ-L-arabinofuranosyl-4-nitrocatechol-1-yl-2-acetate being obtained (2.71 g; 5.95 mmol; 38 %).

α-L-Arabinofuranosyl-4-nitrocatechol-1-yl and β-L-arabinofuranosyl-4-nitrocatechol-1-yl

Preparative TLC was found by us to be an efficient, rapid and convenient method for diastereomers separation. Tri-O-acetyl-αβ-L-arabinofuranosyl-4-nitrocatechol-1-yl-2-acetate (1.5 g; 3.29 mmol) was submitted to Zémplen hydrolysis by incubation with 0.2 M sodium methoxide followed by removing of the excess of alkalinity by stirring with Dowex 50 WX2 (H^+) and filtration. The filtrate was evaporated to dryess and brought to constant weight in a dessicator to obtain a mixture of α- and β-L-arabinofuranosyl-4-nitrocatechol-1-yl (0.875 g; 3.05 mmol; 93 %). A concentrated solution of the mixture of the two diastereomers was made in water-ethanol 1:1 (v/v), applied on preparative plates (20 x 20 cm) and migrated with SS 1. The plates were visualized by UV light, marked, scraped and the silica gel washed in a small column with water-ethanol 1/1 (v/v). Dozens of mg have been obtained of the two diastereomers. They were peracetylated and their NMR spectra registered. The alpha isomer (Fig. 1) had the following characteristics:

^1H NMR. ($CDCl_3$; δ ppm; *J* Hz) 4.92 (s, 1H) (H-1, alpha linkage); 5.06 (1H) (H-2); 5.03 (1H) (H-3); 4.97 (1H) (H-4); 4.40 (1H) (H-5a); 4.21 (1H) (H-5b); 7.20 (d, 8.8 Hz, 1H) (H-3'); 7.93 (1H) (H-5'); 8.07 (1H) (H-6'); 2.01, 2.05, 2.10, 2.40 (s) (Me groups of Ac linked to sugar and 4-nitrocatechol).

^{13}C NMR. ($CDCl_3$; δ ppm; *J* Hz) (fig. 1): 106.7 (C-1); 81.2 (C-2); 70.3 (C-3); 77.1 (C-4); 63.3 (C-5); 152.9 (C-1'); 146.2 (C-2'); 111.0 (C-3'); 145.4 (C-4'); 118.3 (C-5'); 123.3 (C-6'); 20.3, 20.5, 20.7, 20.8 (Me groups of Ac linked to sugar and 4-nitrocatechol); 169.5, 169.9, 170.3, 170.6 (>C=O groups of Ac).

The beta isomer (fig.1) had the following characteristics:

^1H NMR. ($CDCl_3$; δ ppm; *J* Hz) 5.037 (d, 3.6 Hz, 1H) (H-1, beta linkage); 4.217 (1H) (H-2); 5.327 (1H) (H-3); 5.172 (1H) (H-4); 4.001 (1H) (H-5a); 3.603 (1H) (H-5b); 7.229 (1H) (H-3'); 7.954 (1H) (H-5'); 8.120 (1H) (H-6'); 1.999, 2.062, 2.089, 2.330 (s) (Me groups of Ac linked to sugar and 4-nitrocatechol).

^{13}C NMR. ($CDCl_3$; δ ppm; *J* Hz): 105.43 (C-1); 80.01 (C-2); 76.01 (C-3); 68.71 (C-4); 63.19 (C-5); 154.28 (C-1'); 147.96 (C-2'); 110.87 (C-3'); 144,82 (C-4'); 118.50 (C-5'); 123.09 (C-6'); 20.22, 20.67, 20.70, 20.93 (Me groups of Ac linked to sugar and 4-nitrocatechol); 168.15, 169.70, 170.23, 170.42 (>C=O groups of Ac).

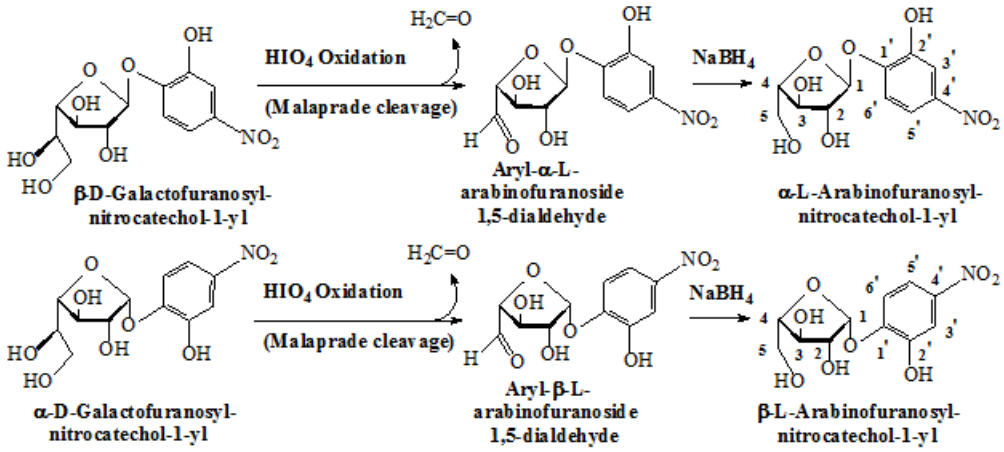

Fig. 1. Redox conversion of β- and α-D-galactofuranosides to α- and β-L-arabinofuranosides, respectively

Periodic acid oxidation (Malaprade reaction) of 4-nitrocatechol-galactofuranosides

β- And α-D-galactofuranosyl-4-nitrocatechol-1-yl have been synthesized as indicated [20,21]. They have been oxidized on lateral chain as indicated [22]: 0.25 g (0.78 mmol) β- or α-D-galactofuranosyl-4-nitrocatechol-1-yl was dissolved in 2 mL of water and to this a solution of 0.334 (1.56 mmol) $NaIO_4$ and 2 mL water was added. The mixture was stirred for 6 hr at room temperature and then 0.2 mL of ethylene glycol was added in order to destroy the unreacted periodate. Then, reduction of the new formed aldehyde was made by adding 0.08 g $NaBH_4$ (2.1 mmol) in small portions, at 0 °C. The mixture was stirred for 6 h at the same temperature and then some acetone was added in order to destroy the unreacted borohydride. The solution was evaporated to dryness and the residue resumed in a small volume of ethanol-water 1/1 (v/v) and separated by preparative TLC (SS 1) and vizualized under UV light. Recovery of glycosides was 65-70 %.

Arabinofuranosides prepared by redox reactions comigrated with directly synthesized arabinofuranosides, in the four SS mentioned above. All 4-nitrocatechol arabinofuranosides were cleaved by exoglycosidases from radish (*Raphanus sativus* L.) germs. However, α-L-arabinofuranosides was cleaved about five times faster than β-L-arabinofuranoside. This constitutes an indirect proof that 4-nitrocatechol galactofuranosides could be good substrates for galactofuranosidases.

Enzymatic assay

Arabinofuranosides were dissoved in water (5 mM). Biological material was homogenized in acetate buffer (acetic acid 0.03 M, sodium acetate 0.07 M, NaCl 0.05 M, *p*H 5.0) [23] in the ratio 1/10 (w/v) and then centrifuged: the supernatant constituted enzymatic solution. Incubation mixture consisted of 0.25 mL substrate, 0.5 mL buffer, 0.25 mL enzymatic extract. Incubation lasted 30-60 min at 40 °C and then 2 mL of 0.2 M sodium hydroxide was added and the final solution measured at 515 nm. A molar coefficient of 12,670 $cm^2 \times mol^{-1}$ was used for 4-nitrocatechol, in a strongly alkaline environment [15].

Results and discussions

We have adopted an indirect synthesis of the aglycon, consisting in the reaction of 4-nitrophenol with $K_2S_2O_8$ in a strongly alkaline solution (KOH); the main product, 4-nitrocatechol sulfate was hydrolyzed to 4-nitrocatechol by acidic hydrolysis. This method is convenient in spite of its relatively low yield, especially since 4-nitrocatechol sulfate is itself a valuable enzymatic substrate. An alternative to this method, i. e. nitration of catechol, leads to two isomers, 4- and 3-nitrocatechol in a molar ratio of 9:5 [24], and they have to be separated.

We have elaborated a test for monitoring glycosylation reaction of 4-nitrocatechol: reaction mixture is analyzed by TLC and the plates are visualized by exposing to alkaline pH [15,20,21]. The result is that the zones contaning unreacted 4-nitrocatechol veer to red colour while glycosylated 4-nitrocatechol turns yellow. We have used exhaustively this test along all the syntheses in this paper. In fact, glycosylation of 4-nitrocatechol can produce di- [25] or monoglycosylated compound [20,21]. ^1H NMR Spectra of peracetylated diglycosylated conjugate indicate exclusively aliphatic acetate esters [25], while monoglycosylated dihydroxyphenol presents a *downfield* effect due to phenolic acetate ester [15,20,21,26]. We have constantly used this signal [27], in the present paper inclusively, as an indicator for monoglycosylation. The signals for glycosides prepared in this paper varied in the interval 2.33-2.40 ppm (1,2-di-O-Ac-4-nitrocatechol had 2.33 and 2.34 ppm [15]). Moreover, all glycosides synthesized in this paper had a molar ratio of 4-nitrocatechol and sugar of 1:1, as determined by acidic hydrolysis and colorimetric reactions.

There were at least three methods in use for the identification of furanosic ring when NMR was introduced in laboratory analysis: (A) the relative susceptibility of furanosides to acidic *p*H [28,29]; (B) permethylation-hydrolysis-oxidation [30,31]; (C) periodate oxidation [32]. On this basis, ä value of H-1, as well as coupling constants, of methyl pyranosides and furanosides, both in alpha and beta configuration, of some monosaccharides – D-glucose, D-galactose, D-xylose and L-arabinose, were measured [33]. There was a good agreement between our spectral data, both galactopyranosides [15] and furanosides [20,21], and the data from literature.

Galactofuranosides of 4-nitrocatechol synthesized in this paper failed to be hydrolyzed in this form by an enzymatic extract of radish germs. This constituted an indirect proof that they hadn't pyranosic ring, since a β-galacto (pyrano)sidase had been found in this tissue [34]. After their redox conversion to arabinofuranosides, they were hydrolyzed, especially α-L-arabinofuranosidic isomer. Specific activity was of the same order of magnitude as indicated [15].

Both D-galactose and L-arabinose are relatively widespread as furanosides in natural materials. A galactan based on β-D-galactofuranose as monomeric unit was discovered in some molds of *Penicillium* species [35], based on its susceptibility to mineral acids. This characteristic of furanosides, still in use today, had been discovered by E. Fischer as soon as he synthesized the first furanoside, methyl-β-D-glucofuranoside [28, 29]. A systematic search of chemical literature [17,18,20,21] showed that D-galactofuranose, either in configuration β or α, can be found in nature in all common groups of compounds: mono-, oligo-, and polysaccharides, glycosylation reagents (UDP-Galf), glycosphingolipids, gangliosides, glycosterols, glycoglycerolipids, polyprenyl-glycolipids (sesquiterpenglycolipids), resorcinolglycolipids (lecythophorin), glycoproteins (phospholipase C and phytase). The catabolism of furanosides is accomplished by two groups of enzymes: exo-galactofuranosidases and endo-galactofuranosidases. Till now, exo-galacto-furanosidases especially were assayed with either chromogenic substrates based on 4-nitrophenol [36,37] or fluorogenic ones, based on 4-methylcoumarin [38].

Arabinofuranosides possess a more limited diversity. However, it can be found in polysaccharides: arabino-galactan, lipoarabinomannan, glycoconjugates [39]. Arabinoxylans from wheat [40] or sorghum [41] are formed of linear polysaccharidic chain constituted of β1-4-D-Xylp units bearing α-L-Araf units on C-2 and/or C-3 of Xyl units. Exo-arabinofuranosidases were assayed with 4-nitrophenyl glycosides [23] or with 6-bromo-2-naphthyl glycosides [42].

Conclusions

Both modified Helferich and Michael-Mannich glycosylation methods produce glycosides of 4-nitrocatechol that can be used as enzymatic substrates.

H and ^{13}C NMR Spectroscopy constitutes a suitable and rapid method for structural characterization of glycosides.

Enzymatic assay of glycosides is a valuable tool that can run bidireactionally: to detect and investigate

biocatalytic activity or for quantitative determination of metabolites.

References

1.MICHAEL, A., Am. Chem. Soc., **I**, 1879, p. 305.
2.FISCHER, E., MECHEL, v., L., Chem. Ber., **49**, 1916, p. 2813.
3.HELFERICH, B., RUDOLF-PETERSEN, S., Chem. Ber., **68**, 1935, p. 790.
4.AIZAWA, K., Enzymologia **6**, 1939, p. 321.
5.AIZAWA, K., J. Biochem. (Tokyo), **30**, 1939, p. 89.
6.SEIDMAN, M., LINK, K. P., J. Am. Chem. Soc., **72**, 1950, p. 4324.
7.MEAD, J. A. R., SMITH, J. N., WILLIAMS, R. T., Biochem J., **61**, 1955, p. 569.
8.ANDERSEN, F. M., K. R. MARKHAM (eds.), FLAVONOIDS: Chemistry, Biochemistry, and Applications, Taylor & Francis Group, Boca Raton London New York, 2006.
9.NEUBERG, C., WAGNER, J., Biochem. Z., **161**, 1925, p. 492.
10.BABERS, F. H., GOEBEL, W. F., J. Biol. Chem., **105**, 1934, p. 473.
11.LEDERBERG, J., J. Bacteriol., **60**, 1950, p. 381.
12.TALALAY, P., FISHMAN, W. H., HUGGINS, C., J. biol. Chem., **166**, 1946, p. 757.
13.LEABACK, D. H., WALKER, P. G., J. Chem. Soc., 1957, p. 4754.
14.BOROOAH, J., LEABACK D. H., WALKER, P. G., Biochem. J., **78**, 1961, p. 106.
15.IGA, D. P., SCHMIDT, R. R., IGA, S., HOTOLEANU, C. L., DUICA, F., NICOLESCU, A., GITMAN, S. S., Turk. J. Chem., **37**, 2013, p. 299.
16.BÖRJESON, H., JERKEMAN, P., LINDBERG, B., Acta. Chem. Scand., **17**, 1963, p. 1705.
17.IGA, D. P., IGA, S., SCHMIDT, R. R., BUZAS, M. C., Carbohydr. Res., **340**, 2005, p. 2052.
18.IGA, D. P., IGA, S., Open Org. Chem. J. (USA), **2**, 2008, p. 46.
19.ROY, A. B., Biochem. J., **53**, 1953, p. 12.
20.IGA, D. P., IGA, S., NICOLESCU, A., CHIRA, N.-A., Rev. Roum. Chim., **55**, 2010, p. 357.
21.IGA, D. P., IGA, S., VELICU, N., PETRUT, T., CONDUR, N.-D., Roum. Biotechnol. Lett., **15**, 2010, p. 5096.
22.KISHORE, N., SINHA, N., JAIN, S., UPADHAYAYA, R. S., CHANDRA, R., ARORA, S. K., ARKIVOC, **I**, 2005, p. 65.
23.HATA, K., TANAKA, M., TSUMURAYA, Y., HASHIMOTO, Y., Plant Physiol., **100**, 1992, p. 388.
24.BOESEKEN, J., MIJS, J. A., Rec. Trav. Chim. **44**, 1925, p. 758.
25.KROGER, L., THIEM, J., Carbohydr. Res. **342**, 2007, p. 467.
26.CEPANEC, I., LITVIÆ, M., ARKIVOC, **II**, 2008, p. 19.
27.DUICA, F., GITMAN, S. S., HOTOLEANU, L. C., NICOLESCU, A., IGA, D. P., Rev. Chim.(Bucharest), **66**, no. 11, 2015, p. 1747.
28.FISCHER, E., Chem. Ber., **26**, 1893, p. 2400.
29.FISCHER, E., Chem. Ber., **47**, 1914, p. 1980.
30.HAWORTH, W. N., RUELL, D. A., WESTGARTH, G. C., J. Chem. Soc., **125**, 1924, p. 2468.
31.HAWORTH, W. N., HIRST, E. L., JONES, D. I., WOODWARD, H., J. Chem. Soc., 1938, p. 1575.
32.JACKSON, E. L., HUDSON, C. S., J. Am. Chem. Soc. 59, 1937, p. 994.
33.CAPON, B., THACKER, D., Proc. chem. Soc., 1964, p. 369.
34.SEKIMATA, M., OGURA, K., TSUMURAYA, Y., HASHIMOTO, Y., YAMAMOTO, S., Plant Physiol., 1989, 90, p. 567.
35.CLUTTERBUCK, P. W., HAWORTH, W. N., RAISTRICK, H., SMITH, G., STACEY, M., Biochem. J., 1934, 28, p. 94.
36.VARELA, O., MARINO, C., DE LEDERKREMER, R. M., Carbohydr. Res., 155, 1986, p. 247.
37.COUSIN, M. A., NOTERMANS, S., HOOGERHOUT, P., VAN-BOOM, J. H., J. Appl. Bacteriol., 66, 1989, p. 311.
38.MARINO, C., CANCIO, M. J., VARELA, O., DE LEDERKREMER, R. M., Carbohydr. Res., 276, 1995, p. 209.
39.BERG, S., KAUR, D., JACKSON, M., BRENNAN, P. J., Glycobiol., 17, 2007, p. 35R.
40.GRUPPEN, H., KORMELINK, F. J. M., VORAGEN, A. G. J., J. Cereal Sci., 18, 1993, p. 111.
41.VERBRUGGEN, M.A., BELDMAN, G.,VORAGEN, A. G. J., Carbohydr. Res., 306, 1998, p. 275.
42.YOSHIDA, S., KAWABATA, Y., KANEKO, S., NAGAMOTO Y., KUSAKABE, I., Biosci. Biotech. Biochem., 58, 1994, p. 580

Manuscript received: 9.11.2015