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ORIGINAL PAPER

New assay of α-L-rhamnosidase

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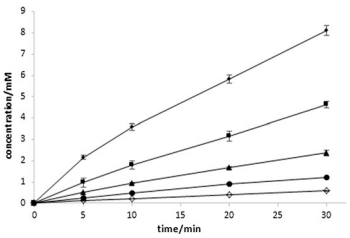
Abstract Free rutinose was prepared by enzymatic hydrolysis of rutin using defatted seed meal from tartary buckwheat. This disaccharide was used as substrate in spectrophotometric assay of α-L-rhamnosidase. The assay is based on hydrolysis of rutinose and subsequent determination of released glucose by a standard glucose oxidase assay kit. The method is easy to perform and requires no expensive equipment. The assay was applied in α-L-rhamnosidase estimation in ten commercial enzyme preparations and compared with standard assay on chromogenic substrate.

Graphical abstract



Keywords Carbohydrates · Enzymes · Hydrolysis · Oxidation · Rhamnosidase

Introduction

α-L-Rhamnosidases (E.C. 3.2.1.40) are glycosidases which cleave terminal α-L-rhamnose from a great variety of natural compounds such as pectins, O-antigen polysaccharides, and rhamnolipids [1–3] as well as various less complex natural glycosides like rutin, quercitrin, naringin, hesperidin, and saponin rhamnosides [4–7]. This type of enzymes can be found among bacteria, yeasts, fungi, plants, and animals, and α-L-rhamnosidase activity frequently occurs within enzyme complexes together with the β-D-glucosidase activity, e.g., hesperidinase [6], naringinase [7], and rhamnodiastase [8]. Rhamnosidases are frequently used in food industry for debittering of citrus fruit juices and prevention of their turbidity caused by precipitation of hesperidin [9, 10] or in aroma release in beverages [11].

4-Nitrophenyl α-L-rhamnopyranoside (**1**) is commercially available but relatively expensive substrate for α-L-rhamnosidase assay. Although its preparation has been reported [12], the compound is not accessible without experience with techniques of organic synthesis. Moreover, determination of α-L-rhamnosidase activity exclusively with chromogenic substrates does not offer the proper insight into the enzyme specificity, and therefore, it is necessary to perform other assays to uncover affinity to given natural substrates, such as molecules harboring rutinose motif [13].

Naringin can be used as the natural substrate, but disadvantage of its use is in the reaction complexity: the method follows overall disappearance of the substrate

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naringin and intermediate prunin at the same time and additional testing is necessary to distinguish between these two reactions [14]. The reaction can be obviously followed by HPLC, but this is not supposed to be a fast way. Another option is to use commercial rhamnose dehydrogenase kits to follow the released rhamnose photometrically, but this method is also costly, although it can be universally modified to virtually any rhamnoside substrate. Therefore, there still exists a demand for robust, fast, and economic rhamnosidase assay.

Rutinose (6-*O*-α-L-rhamnopyranosyl-D-glucopyranose, **2**) is a disaccharide with potential medicinal applications referred to the L-rhamnose moiety [15] and it may serve also as a natural substrate for assay of α-L-rhamnosidase. Its chemical synthesis is known but is difficult to accomplish. Contrariwise, fruit processing side products are inexpensive and easily available sources of rutin (quercetin 3-*O*-β-rutinoside, **3**) and hesperidin which can be hydrolyzed to rutinose in quality sufficient for non-medical uses. Hydrolysis of rutin was earlier performed, for example, by rhamnodiastase from leaves of *Rhamnus californica* in 1962 [16].

Rutinosidase (EC 3.2.1.168) is a diglycosidase specific to hydrolysis of rutinosides liberating the whole disaccharide and the free aglycon. Rutinosidases hydrolyse rutin and hesperidin; in some cases, they hydrolyze also naringin or they may possess β-glucosidase activity towards monoglucosides [17]. Application of these diglycosidases is not limited to liberation of aroma in food production, but there is growing importance of their use in the synthesis of rutinosides [18].

The purpose of this paper is to introduce simple assay of α-L-rhamnosidase based on hydrolysis of rutinose by rhamnosidase and to demonstrate preparation of the necessary substrate rutinose through hydrolysis of rutin, using techniques available in every chemical or biochemical laboratory.

Results and discussion

Preparation of rutinose

Rutinose is available on the market in prices which are unacceptable for routine enzyme assays. It was, therefore, prepared in our laboratory by hydrolysis of rutin (**3**) catalyzed by rutinose in seed meal from tartary buckwheat (*Fagopyrum tataricum*, Scheme 1). Such hydrolysis together with transrutinosylations was reported by several authors [19–23], but it has never been used for gram scale preparation of rutinose.

Although rutinosidase was found in several sources of plant [24–26] or microbial origin [17, 27–30], seeds of tartary buckwheat were found as a practical material due to their availability and ease of storage and handling. In agreement with findings of Morishita et al. [23], the seeds of common buckwheat *Fagopyrum esculentum* did not release any free rutinose from rutin in our hands. On the other hand, hydrolysis of rutin catalyzed by tartary buckwheat ran to the completion, and besides rutinose, only traces of monosaccharides were detected in the reaction mixture. Using simple isolation and purification procedures, we obtained rutinose as a pale yellow powder with purity above 97% in 64% yield. Zhou et al. [19] isolated rutinose from similar reaction in 92% yield; their product had, however, appearance of yellow gum. Šimčíková et al. [29] prepared rutinose in a reaction catalyzed by recombinant fungal rutinosidase in 63% overall yield as a colorless amorphous solid. These two preparative reactions were, however, carried out in scale of tens of milligrams of product. We have run the hydrolysis in multi-gram scale due to simple and inexpensive isolation techniques which make the product accessible in any chemical or biological laboratory in gram quantities.

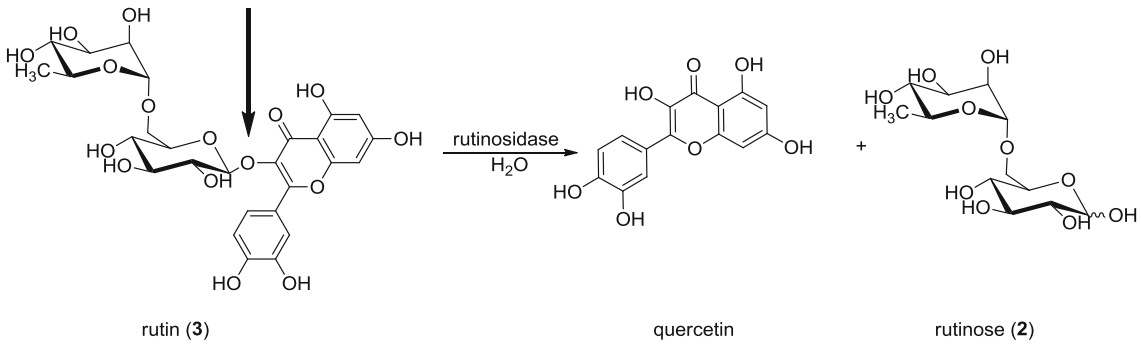
Development of the assay

The principle of this assay is notoriously known from enzymatic estimations of disaccharides such as maltose, lactose, or sucrose or from assays of corresponding glycosidases [31–34]. Rutinose is hydrolyzed to rhamnose and glucose. Portions of reaction mixture are periodically withdrawn, stopped by mixing with methanol, and glucose enzymatically released from the disaccharide is estimated by its oxidation with glucose oxidase. Hydrogen peroxide, an equimolar by-product of this oxidation, oxidizes a phenolic substance (3-methylphenol in our case) in the presence of peroxidase and 4-aminoantipyrine to form quinone–imine colored product (Scheme 2). BIO-LA-TEST® Glucose, an enzymatic test for estimations of glucose in medicine, was used as an inexpensive and reliable ready-to-use kit for glucose determination in the assay.

Several precautions had to be, however, taken into account during development of the assay including substrate specificity of rhamnosidase (acceptation of rutinose as substrate of rhamnosidase), substrate specificity of glucose oxidase (potential oxidation of rutinose), interferences of phenolics in rutinose (rutin and quercetin as potential impurity) and occurrence of glucose or glucose precursor in the tested rhamnosidase.

Two enzymes were preliminary tested in the proposed assay—prokaryotic α-L-rhamnosidase from Megazyme and Viscozyme L, a glycanase from *Aspergillus aculeatus* which was ultrafiltered and lyophilized before testing to remove sugars, salts, and preservers from its original preparation. The results were compared with standard

Scheme 1



Scheme 2

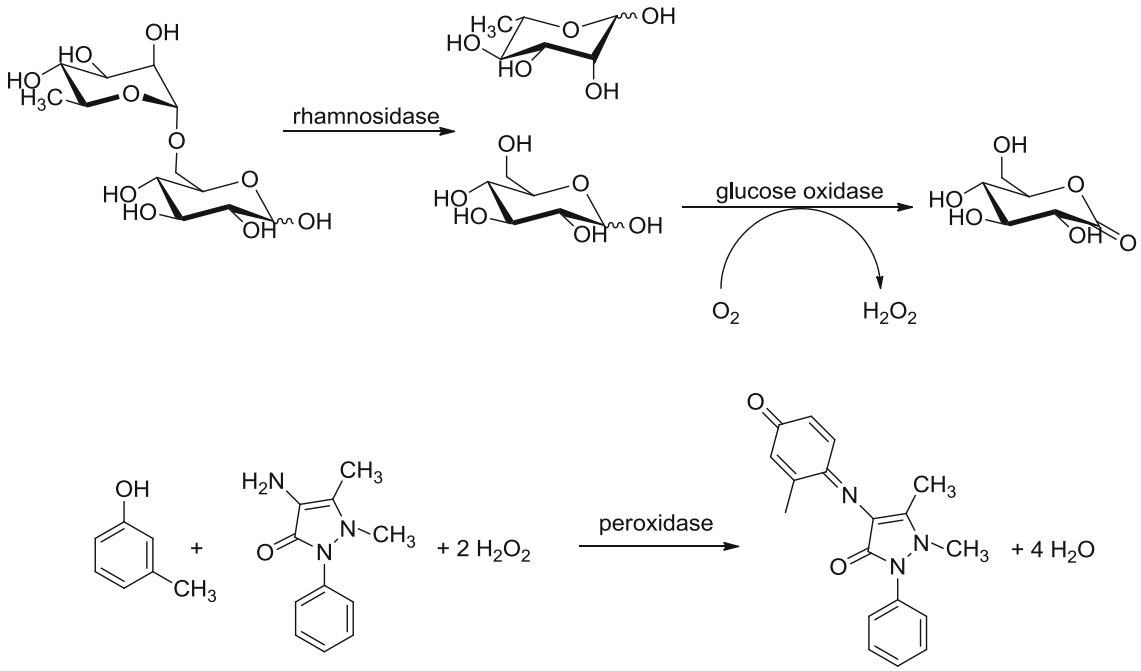


Table 1 α-L-Rhamnosidase activity towards substrates **1** and **2** at pH 6.0

Enzyme	Activity	
	1	**2**
Prokaryotic rhamnosidase	260.3 ± 35.7[a]	1.2 ± 0.3[a]
Viscozyme L (lyoph.)	14.3 ± 1.0[b]	67.1 ± 2.1[b]

[a] Activity in U cm^{-3}

[b] Activity in U g^{-1}

rhamnosidase assay using *p*-nitrophenyl α-L-rhamnopyranoside (Table 1). Activity assays were performed at pH 6.0, according to recommendations from producer of the prokaryotic α-L-rhamnosidase. This latter enzyme has significantly higher affinity towards the synthetic substrate. On the contrary, Viscozyme L shows opposite substrate preference, although the difference is less dramatic (almost five time higher activity on rutinose). According to the literature, differences in specificity towards natural and synthetic substrates are rather frequent. As an example, Grandits et al. [13] reported that the difference in specificity towards natural and synthetic substrate enabled resolution of two distinct α-L-rhamnosidases from the same source.

Optimization of the assay

Ultrafiltered and lyophilized preparation of Viscozyme L was selected for further optimization of the assay as an inexpensive enzyme model with good response to rutinose. Viscozyme L is a fungal enzyme and fungal glycosidases have typically pH optima within the interval 4.5–5.5. Profiles of optimal pH of rhamnosidase activity in Viscozyme L on both substrates similarly peaked at pH 5, although the profile of rutinose hydrolysis had sharper decline of activity outside this pH value (Fig. 1). Obviously, pH 5.0 was used in further α-L-rhamnosidase assays.

Examination of the rate of hydrolysis of rutinose as a function of its initial concentration revealed that the velocity reached its plateau at 139.3 mM (Fig. 2). The initial concentration of rutinose 139.3 mM (corresponding to 50 mg cm^{-3} substrate solution before mixing with the enzyme) was then standardly used in assays of rhamnosidase.

The relation between enzyme concentration and kinetics of rutinose hydrolysis was tested in interval of Viscozyme concentrations 0.23–3.64 mg cm^{-3}. Enzyme loads above 0.9 mg cm^{-3} (corresponding to rhamnosidase activity 0.1 U cm^{-3}) markedly shortened the interval of linear course of rutinose hydrolysis (Fig. 3), and therefore, it is preferable to stop the reaction after 5 min. Using 5 min hydrolysis, the relation between velocity of hydrolysis and enzyme load was linear in the tested interval of Viscozyme concentrations (Fig. 4).

Interferences and precautions

To develop a reliable rhamnosidase assay, several issues had to be resolved to eliminate interferences and error readings coming from the fact that the method is based on estimation of glucose. The response to glucose may vary depending on the storage of the working solution. Standard of glucose must be, therefore, estimated together with every assay. The enzymatic reaction in the withdrawn sample must be immediately stopped to correctly estimate the amount of produced glucose within time intervals. Mixing the sample with three volumes of methanol and immediate vortexing inhibited the tested enzymes. This was proven by stability of final absorbance reads after mixing with glucose oxidase solution. No inhibition effect of methanol in the sample on glucose oxidase test was observed. Obviously, methanol contents in the glucose standard and in real samples were kept identical.

Glucose oxidase is known for negligible oxidation of several other sugars than glucose [35]. Moreover, the working solution of rutinose may contain traces of glucose either as impurity from its synthesis or from spontaneous hydrolysis of the disaccharide if low pH is necessary for

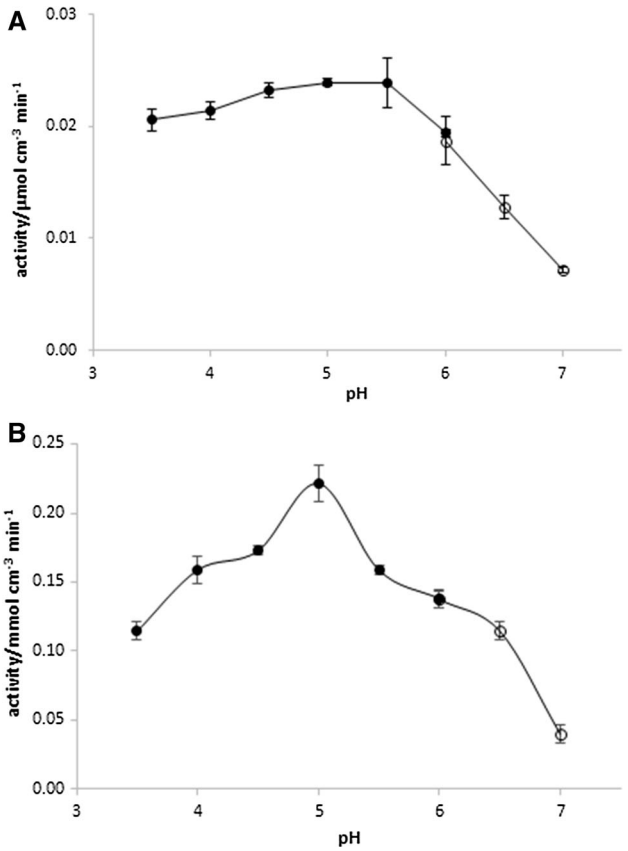


Fig. 1 Activity of α-L-rhamnosidase in Viscozyme L as a function of pH determined with *p*-nitrophenyl α-L-rhamnoside (**a**) and rutinose (**b**). Reaction mixture A (1 cm^3, 40 °C) comprised 0.25 mg of lyophilized Viscozyme in 4.75 mM solution of the *p*NP α-L-rhamnopyranoside in 0.95 M acetate buffer, pH 5.0. The reaction mixture B (1.1 cm^3, 40 °C) comprised 2 mg of lyophilized Viscozyme and 50 mg rutinose in 0.09 M acetate (closed circle) or 0.09 M phosphate (open circle) buffer

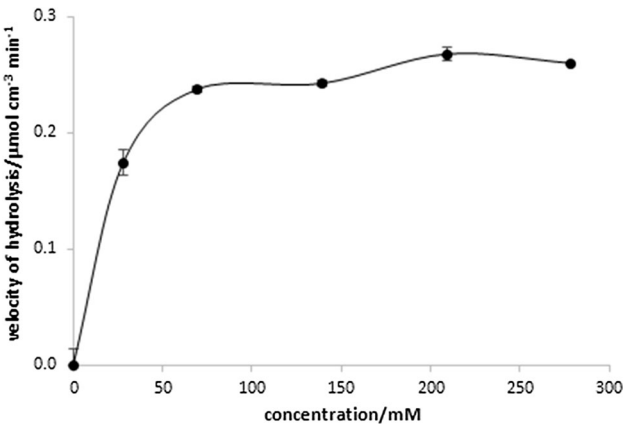


Fig. 2 Effect of rutinose concentration on velocity of its hydrolysis by Viscozyme L. Incubations were carried at 40 °C in 0.1 M acetate buffer pH 5.0 containing 27.9–278.6 mM rutinose and 1.82 mg cm^{-3} lyophilized Viscozyme

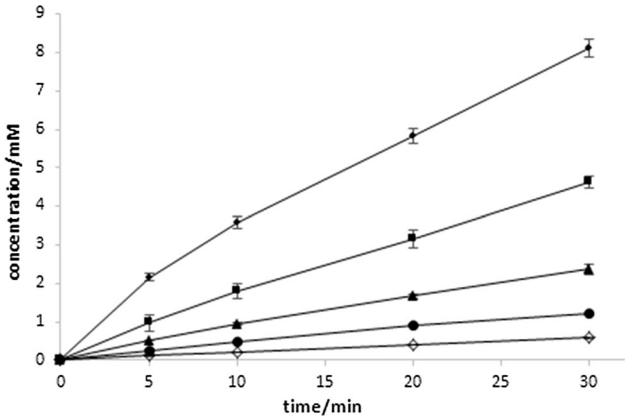


Fig. 3 Time course of the hydrolysis of rutinose expressed as concentration of released glucose. Incubations were carried at 40 °C in 0.1 M acetate buffer pH 5.0 containing 139.3 mM rutinose and lyophilized Viscozyme in amounts: 0.23 mg cm^{-3} (open diamond), 0.45 mg cm^{-3} (closed circle), 0.91 mg cm^{-3} (closed triangle), 1.82 mg cm^{-3} (closed square), and 3.64 mg cm^{-3} (closed diamond)

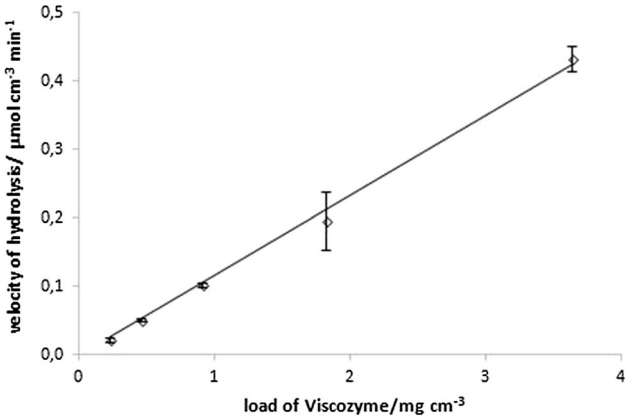


Fig. 4 Velocity of rutinose hydrolysis after 5 min as a function of load of Viscozyme L. Incubations were carried at 40 °C in 0.1 M acetate buffer pH 5.0 containing 139.3 mM rutinose

rhamnosidase assay. Responses corresponding to the level of glucose 0.26–0.28 mM over the pH range 3.5–7.0 were observed for samples containing 139.3 mM rutinose (Fig. 5). The stabilized absorbance reading did not rise up in the time interval between 5 and 60 min, i.e., rutinose itself is not substrate of glucose oxidase. The mentioned negligible responses (below 0.2%) come probably from traces of glucose present as impurity in the substrate. In case of such low background response of pure substrate, the blank of rutinose may be omitted.

Another precaution must be taken when commercial enzymes comprising oligosaccharides as bulk extenders such as lactose, starch, etc. are to be assayed. In such case, the enzyme blank must be processed by exactly the same method as the sample, including the stopping of the reaction after identical time interval. Although free glucose can

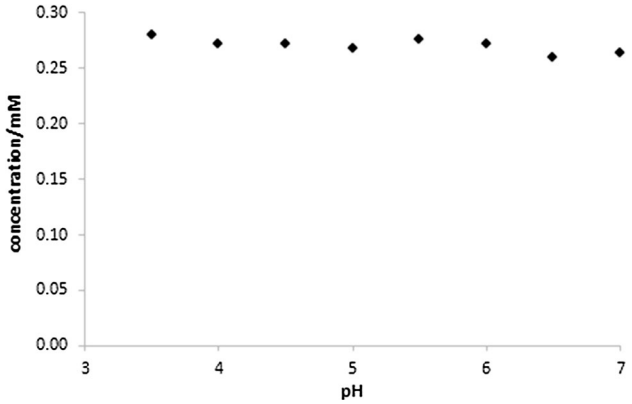


Fig. 5 Response of glucose oxidase test to 139.3 mM rutinose in varying pH (expressed as concentration of glucose)

be estimated directly, the bulk extenders may stepwise hydrolyze after dissolving in assay mixture if a glycosidase with respective activity is present. For example, commercial pectinase Lallzyme Cuveé Blanc comprises low rhamnosidase activity according to the assay with *p*NP rhamnoside. When the enzyme was dissolved in buffer with no rutinose added, gradual release of glucose occurred (Fig. 6). On contrary, enzyme inactivated after 10 min comprised only starting glucose level which did not rise up during the further incubation. This is a proof that the enzyme preparation itself can be source of gradual glucose release, thus imitating rutinose hydrolysis. Such situation may lead either to false positive results in rhamnosidase screenings or to high background glucose hampering estimation of low level of rhamnosidase. It is, therefore, necessary to provide enzyme blanks (without rutinose) within the assay.

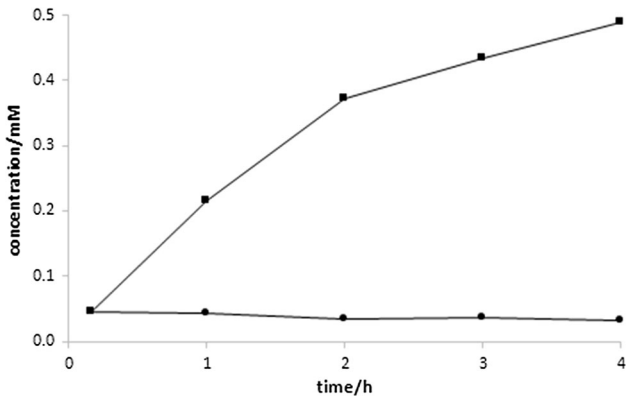


Fig. 6 Development of glucose in solution of non-denatured (closed square) and methanol-denatured (closed circle) Lallzyme Cuveé Blanc (2 mg cm^{-3}) in 0.1 M acetate buffer pH 5.0

α-L-Rhamnosidase assays in real samples

Preparations of ten commercial enzymes of microbial origin were assayed for α-L-rhamnosidase activity both on rutinose and on 4-nitrophenyl α-L-rhamnopyranoside (*p*NP Rhap). Obviously, nominal values of rhamnosidase activity measured on the two substrates were not identical. Samples possessing moderate levels of rhamnosidase on the synthetic substrate displayed three-to-four time higher nominal activity in assays on rutinose (Table 2). The assay on the synthetic substrate was, however, more sensitive in tests of samples comprising low rhamnosidase and high glucose background. The method had detected rhamnosidase activity under 5 U g^{-1} of the enzyme, while assays of the same samples on rutinose displayed zero or low response.

Some exception in reaction behavior was observed for rhamnosidase in α-galactosidase DS from Amano. Its response to rutinose was more than 18 times higher over the response to *p*NP Rhap suggesting strong preference of this enzyme to non-glycosylated rhamnosaccharides.

Conclusion

We report a new photometric assay of α-L-rhamnosidase based on hydrolysis of rutinose. Although the assay has a limited application in screenings among commercial enzymes with high content of glucose or glucose precursor, it may find use in routine assays of rhamnosidases with proven specificity towards rutinose provided that a set of blanks is prepared together with the enzyme sample. The substrate is easy to prepare in any chemical or biochemical laboratory in gram quantities by simple, inexpensive procedures.

Experimental

Catalysts and chemicals

Seeds of common buckwheat (*Fagopyrum esculentum*) were purchased from a local supplier. Seeds of *Fagopyrum tataricum* var. Madawaska were from the seed bank of the National Agricultural and Food Centre, Research Institute of Plant Production Piešťany. High-purity recombinant α-L-rhamnosidase (prokaryote, EC 3.2.1.40) was purchased from Megazyme International (Ireland). Rapidase AR-2000 was kind gift from Patrice Pellerin (DSM, USA), Lallzyme BETA and Lallzyme Cuvée Blanc were purchased from Lallemand (Canada). Lypolyve AN was from Lyven (France), Enzeco Microbial Lipase was a gift from Enzyme Development Corporation (USA) and Rohament was a gift from Barentz Slovakia (local supplier of AB Enzymes GmbH, Germany). Crude enzyme preparations Viscozyme L and Novozym 188 were a kind gift from Marián Illáš (Biotech s.r.o., Slovakia) and were ultrafiltered and lyophilized prior use. BIO-LA-TEST Glucose® kit is product of Erba Lachema (Brno, Czech Republic). Celite® 545 was purchased from Alfa Aesar GmbH Co KG (Germany). Powdered activated charcoal was purchased from Mikrochem s.r.o. (Slovakia). TLC standards: anhydrous D-glucose was purchased from Mikrochem s.r.o. (Slovakia), L-rhamnose 99%, rutin hydrate ≥94% (HPLC), and quercetin hydrate ≥95% (HPLC) were purchased from Sigma-Aldrich (USA). All other commercial reagents and solvents used in experiments were purchased locally in satisfactory purity (min. 95%).

Analytical methods and other apparatus

Spectrophotometric determination of liberated glucose was performed on UV–Vis Spectrophotometer UV-1800 (Shimadzu, Kyoto, Japan). ^{1}H NMR (600 MHz) and ^{13}C NMR

Table 2 α-L-Rhamnosidase activity towards substrates **1** and **2** at pH 6.0

Enzyme	Activity/U g^{-1}	
	1	**2**
Viscozyme L (lyoph.)	25.1 ± 0.1	81.3 ± 4.8
Rapidase AR 2000	7.1 ± 0.8	29.6 ± 3.4
Enzeco lipase	9.9 ± 0.3	34.4 ± 3.4
Novozym 188 (lyoph.)	14.6 ± 1.0	50.4 ± 8.2
Amano lipase A	12.1 ± 0.4	33.9 ± 2.7
Lallzyme BETA	2.5 ± 0.0	3.9 ± 1.4
α-Galactosidase DS	6.3 ± 0.2	119.2 ± 1.4
Ultrazym 100	0.9 ± 0.1	n.d.
Lallzyme Cuvée Blanc	1.6 ± 0.0	n.d.
Lipolyve AN	4.5 ± 0.4	n.d.

(151 MHz) spectra were recorded with a Varian 600 NMR spectrometer. Spectra were taken with about 100 mM solution in D_2O.

Incubations in small scale were performed in Eppendorf tubes on Thermomixer comfort (Eppendorf AG, Hamburg, Germany). Incubations in preparative scale were performed in Thermobox KS 4000 ic control from IKA (Staufen, Germany). Ultrafiltration was performed on filter VIVA-FLOW 200 (10,000 MWCO PES) purchased from Sartorius Stedim Biotech (GmbH, Göttingen, Germany) with peristaltic pump Masterflex L/S (Cole-Parmer International, Vernon Hills, IL, USA). Lyophilization of the ultrafiltered enzymes was performed on freeze dry system LABCONCO Freezone 18 overnight. Optical rotations were measured with a Perkin-Elmer 241 polarimeter at 20 °C.

TLC alumina plates Silica gel 60 F_{254} from Merck KGaA (Darmstadt, Germany) were used for following the process of rutin hydrolysis by TLC. Plates were eluted by chloroform/methanol/water (12/7/1, v/v/v). The spots were detected by charring the plates with 5% (v/v) ethanolic solution of H_2SO_4 and heating at ca. 200 °C.

High-performance liquid chromatography was performed on an Agilent 1200 Series apparatus consisting of quarternary pump, RI and UV–Vis detectors, column thermostat, and a Rheodyne injector with a 20 mm^3 loop. Purity of rutinose was estimated on Polymer IEX H^+ column (250 × 8 mm) from Watrex eluted with 9 mM H_2SO_4 (1 cm^3 min^{-1}) at 35 °C with detection by RI detector.

Preparation of rutinose

Seeds of tartary buckwheat were homogenized in coffee grinder. Scaly parts were then separated on 1 mm screen and the meal was extracted by ethyl acetate in Soxhlet extractor for 48 h to remove fat and soluble substances. Dried crude flour was then homogenized by mortar and pestle and sieved through 0.3 mm screen. This material was then stored in dark and dry conditions for further use.

Free rutinose was prepared by hydrolysis of rutin. As an example, 5 g rutin (8.2 mM) was dissolved in 1 dm^3 of distilled water and 2.5 g of powdered tartary buckwheat meal was added into the reaction. Hydrolysis ran for 3 h at 40 °C and 150 rpm. Solids were then separated from the reaction mixture by vacuum filtration and the filtrate was concentrated on rotary vacuum evaporator to volume about 20–30 cm^3. The concentrate was applied on Biotage SNAP cartridge filled with slurry of 50 g mixture of activated charcoal and Celite® 545 (1:1, w/w) pre-equilibrated with 10 column volumes of distilled water. Monosaccharides were eluted from the column with 1.2 dm^3 of water. Fractions of about 150 cm^3 were continuously collected, evaporated, and analyzed by TLC. Column was then subsequently eluted with gradient of ethanol in water (800 cm^3 of 2%, 800 cm^3 of 4% and 800 cm^3 of 8% ethanol in water). Fractions containing pure disaccharide were combined, concentrated on rotavap, and lyophilized overnight to give 1.7 g (64%) of pale yellow powder with purity above 97% (HPLC). We have prepared, overally, four different batches in scales from 1 to 5 g and the chemical yields of pure rutinose were always above 60%. $R_f = 0.15$ (glucose = 0.24, rhamnose = 0.46) (chloroform/methanol/water, 12:7:1, v/v/v); $[\alpha]_D^{20} = -6.0$ (c = 1.0, H_2O, 24 h equil.) [Ref. [36] $[\alpha]_D^{20} = -10$ (c = 1.0, EtOH), Ref. [19] $[\alpha]_D^{25} = -0.5$ (c = 0.00834, H_2O)]. The product decomposed at 182–189 °C [Ref. [37] 185–189 °C (decomposition)]. Structure of the product was verified by ^1H and ^{13}C NMR (see supplementary file) and chemical shifts of atoms agree with data published previously by Roslund et al. [38]. The lyophilized powder was then used for activity assay.

α-L-Rhamnosidase assays

Assay with rutinose: rutinose (50 mg in 1 cm^3 0.1 M acetate buffer pH 5.0 unless otherwise stated) was preheated at 40 °C and mixed with 100 mm^3 of α-L-rhamnosidase solution. After 5 min, predefined volume of reaction mixture was withdrawn and mixed with three volumes of methanol and immediately vortexed to stop the reaction. The methanolic solution (100 mm^3) was then added to 1 cm^3 of pre-tempered working solution of the glucose oxidase kit. The mixture was incubated for 15 min at 37 °C along with the standard comprising 100 mm^3 glucose (0.55 mM in methanolic buffer) and 1 cm^3 working solution. The absorbance at 498 nm was read not later than after 40 min. Solution comprising corresponding concentration of rutinose without enzyme and a solution with corresponding amount of enzyme without rutinose were used as blanks. All blanks were processed the same way as the hydrolytic reaction, including time intervals and mixing with methanol.

Concentration of glucose was calculated according to the following equation:

$$c_{glc} = c_{st} \times \frac{A_s - A_{eb} - A_{rb}}{A_{st}}, \quad (1)$$

where c_{glc} stands for concentration of glucose in the sample and c_{st} for concentration of glucose in the standard. A_s, A_{eb}, A_{rb}, and A_{st} stand for absorbances of sample, enzyme blank, rutinose blank, and standard glucose solution, respectively.

One unit of enzyme activity was defined as the amount of enzyme catalyzing releasing 1 μmol of glucose min^{-1} under the described conditions. Within optimization experiments, conditions of assay were varied according to

rutinose concentration, buffer type and pH, enzyme load, and time of hydrolysis.

Assay with *p*NP α-L-rhamnopyranoside: an amount of 50 mm^3 of diluted enzyme was mixed with 0.95 cm^3 of 5 mM solution of the *p*NP α-L-rhamnopyranoside in 0.1 M acetate buffer, pH 5.0. Reaction mixture was incubated for 5 min at 1000 rpm and 40 °C. The enzymatic reaction was terminated by the addition of six volumes of saturated solution of borax. For determination of spontaneous hydrolysis of glycosides, 50 mm^3 of buffer was added instead of the enzyme sample and used as blank value. The formation of *p*-nitrophenol was measured at 410 nm and its respective amount was calculated from the calibration curve. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of *p*-nitrophenol per minute under the described conditions.

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