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Dual role of imidazole as activator/inhibitor of sweet almond (*Prunus dulcis*) β-glucosidase

Sara Caramia[a], Angela Gala Morena Gatius[a], Fabrizio dal Piaz[b], Denis Gaja[a], Alejandro Hochkoeppler[a,c,*]

[a] Department of Pharmacy and Biotechnology, University of Bologna, Viale Risorgimento 4, 40136 Bologna, Italy
[b] Department of Medicine, University of Salerno, Via Giovanni Paolo II 132, 84084 Fisciano, SA, Italy
[c] CSGI, University of Firenze, Via della Lastruccia 3, 50019 Sesto Fiorentino, FI, Italy

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ABSTRACT

The activity of *Prunus dulcis* (sweet almond) β-glucosidase at the expense of *p*-nitrophenyl-β-D-glucopyranoside at pH 6 was determined, both under steady-state and pre-steady-state conditions. Using crude enzyme preparations, competitive inhibition by 1–5 mM imidazole was observed under both kinetic conditions tested. However, when imidazole was added to reaction mixtures at 0.125–0.250 mM, we detected a significant enzyme activation. To further inspect this effect exerted by imidazole, β-glucosidase was purified to homogeneity. Two enzyme isoforms were isolated, i.e. a full-length monomer, and a dimer containing a full-length and a truncated subunit. Dimeric β-glucosidase was found to perform much better than the monomeric enzyme, independently of the kinetic conditions used to assay enzyme activity. In addition, the sensitivity towards imidazole was found to differ between the two isoforms. While monomeric enzyme was indeed found to be relatively insensitive to imidazole, dimeric β-glucosidase was observed to be significantly activated by 0.125–0.250 mM imidazole under pre-steady-state conditions. Further, steady-state assays revealed that the addition of 0.125 mM imidazole to reaction mixtures increases the K_m of dimeric enzyme from 2.3 to 6.7 mM. The activation of β-glucosidase dimer by imidazole is proposed to be exerted via a conformational transition poising the enzyme towards proficient catalysis.

1. Introduction

According to their catalytic mechanism, β-glucosidases are classified as inverting or retaining glycohydrolases [1–3]. Remarkably, inverting and retaining β-glucosidases share, along their reaction paths, the generation of a transition state characterized by sp^2 hybridization of the glucose C1 [4]. Accordingly, the planar nature of glucose C1 in the transition state is of importance to design enzyme inhibitors.

To date, quite a number of β-glycohydrolases were characterized and classified into different families according to their primary structures. Sweet almond (*Prunus dulcis*) β-glucosidase (EC 3.2.1.21) contains the sequence motif ITENG, which includes a catalytic glutamate (E426), and is diagnostic of family 1 enzymes [5]. This enzyme was shown to feature broad specificity [6,7], and to act according to a retaining mechanism [8]. Moreover, it was recently demonstrated that the two catalytic steps, respectively leading to glycosilated enzyme and to the release of glucose, feature SN2 and SN1 character, respectively [9]. The pH-dependence of sweet almond β-glucosidase activity is bell-shaped [7,10], suggesting the titration of the two acidic residues involved in catalysis. The pH optimum is centered at 5.6 [10,11], and the two pKa values accordingly determined are equal to 4.4 and 6.7 [7,10]. These values can be reasonably assigned to the nucleophile (E426) and to the acid/base catalytic residue, respectively. Interestingly, a detailed and elegant inspection of the effects, if any, of ionic strength on the sweet almond enzyme revealed that both NaCl and LiCl decrease and increase the acidity of E426 and of the acid/base catalytic residue, respectively [12]. In particular, the decrease of E426 pKa induced by ionic strength suggests a non trivial effect, e.g. that the two catalytic residues interact and mutually affect their acid/base properties [12].

The broad specificity of sweet almond β-glucosidase is easily explained by the rather small differences between the k_{cat}/K_m second-order rate constants observed in the presence of *p*-nitrophenyl derivatives of fucose, glucose, and galactose [7]. The same poor specificity holds when the aglycone is considered: about 20 different derivatives of glucose were shown to be susceptible of cleavage by the sweet almond

* Corresponding author at: Department of Pharmacy and Biotechnology, University of Bologna, Viale Risorgimento 4, 40136 Bologna, Italy.
E-mail address: a.hochkoeppler@unibo.it (A. Hochkoeppler).

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enzyme. However, in this case the k_{cat}/K_m ratio varies over three orders of magnitude, depending on the pKa of the aglycone leaving group, the best substrates being those with the lower pKa [7,13].

The finding that some defects of β-glucosidases are linked to human diseases, e.g. the Gaucher syndrome [14], prompted detailed investigations on compounds able to inhibit these enzymes. Early enough, a vast array of potential inhibitors of sweet almond β-glucosidase was tested [7]. In particular, different carbohydrates, lactones, phenols and amines, comprising quite a number of their derivatives, were assayed. Amines and lactones were more effective as inhibitors when compared to carbohydrates and phenols [7]. Later on, imidazole, as well as histidine, histamine, and histidinol [15,16] were found to be potent inhibitors of sweet almond β-glucosidase, with K_i values ranging from 1 to 7 μM (histidinol, histamine, and histidine) to 0.5 mM (imidazole). The action of these compounds as proton buffers, therefore interfering with catalysis, was proposed [16].

Despite the detailed knowledge accumulated on sweet almond β-glucosidase, some relevant points concerning this enzyme were not elucidated yet. In particular, the tertiary structure is not known, and the contribution to observed enzyme kinetics by monomeric and oligomeric forms was not investigated. The primary structure of sweet almond β-glucosidase contains 544 amino acids, yielding a molecular mass equal to 62 kDa. Previous studies have described the native enzyme as a dimer, but the search for activity by a monomeric form was never attempted. Moreover, it was observed that some potent inhibitors of sweet almond β-glucosidase, e.g. nojirimycin, do not trigger linear effects as a function of their concentration. This was explained by conformational changes of the enzyme induced by inhibitor binding [17], similarly to what proposed for substrate binding [18]. To deepen the knowledge about sweet almond β-glucosidase, and the mode of action of enzyme inhibitors, we report here on the activity of crude and highly purified enzyme isoforms. In particular, the kinetics of the reaction at the expense of *p*-nitrophenyl-β-D-glucopyranoside is shown, both under steady-state and pre-steady-state conditions, in the absence or in the presence of imidazole.

2. Materials and methods

2.1. Materials

Lyophilized β-glucosidase from sweet almonds, buffers, and *p*-nitrophenyl-β-D-glucopyranoside (PNPGluc) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Pre-packed HiTrap-Q anion exchange column, and Superdex 200 resin were from GE Healthcare Life Sciences (Piscataway, NJ, USA). Electrophoresis reagents of high purity were purchased from Bio-Rad (Hercules, CA, USA).

2.2. Partial purification of β-glucosidase

The enzyme powder was dissolved in 50 mM Tris-HCl, 150 mM NaCl (pH 8), the solution was centrifuged (10,000 × *g*, 20 min), and the supernatant was loaded onto a Superdex 200 column (1.6 × 70 cm), previously conditioned with 50 mM Tris-HCl, 150 mM NaCl, pH 8. The flow rate was 0.6 mL/min, 0.9 mL fractions were collected, and then subjected to SDS-PAGE to detect the presence of dimeric and monomeric β-glucosidase.

2.3. Purification to homogeneity of β-glucosidase

The enzyme powder was dissolved in 50 mM Tris-HCl (pH 8), the solution was centrifuged (10,000 × *g*, 20 min), and the supernatant was loaded onto a HiTrap-Q (5 mL) column, conditioned with 50 mM Tris-HCl (pH 8). After loading the sample, the column was washed with the equilibration buffer (10 column volumes), and a linear 0–600 mM NaCl gradient was then applied. A consistent fraction of β-glucosidase was eluted with the column wash. This fraction was then concentrated and loaded onto a gel filtration Superdex 200 column (1.6 × 70 cm), previously conditioned with 50 mM Tris-HCl, 150 mM NaCl, pH 8. The flow rate was 0.6 mL/min, 0.9 mL fractions were collected, and then subjected to SDS-PAGE. The fractions containing pure monomeric or dimeric isoforms of β-glucosidase were concentrated with an Amicon ultrafiltration cell, equipped with a YM-30 membrane, and then stored at −20 °C until used. The gel filtration column was calibrated with Low-Molecular-Weight protein standards (GE Healthcare Life Sciences).

2.4. Steady-state activity assays

The activity of sweet almond β-glucosidase was assayed at 20 °C in a universal buffer containing 25 mM each of MES, MOPS, and Tris [19]. The release of *p*-nitrophenol at the expense of PNPGluc was determined spectrophotometrically at 347 nm, using a Cary 300 UV–VIS spectrophotometer. The molar extinction coefficient of *p*-nitrophenol at 347 nm was assumed equal to 3500 $M^{-1}cm^{-1}$ [7].

2.5. Stopped-flow experiments

All measurements were performed using a KinTek (Snow Shoe, PA, USA) SF2004 stopped-flow equipment, and detecting the release of *p*-nitrophenol from PNPGluc at 347 nm. The enzyme syringe contained crude or purified β-glucosidase (in universal buffer, pH 6) at the indicated concentrations, and the second syringe contained 1.1 mM pNPGluc dissolved in universal buffer, pH 6. When present, imidazole (dissolved in universal buffer, pH 6) was added to the enzyme syringe. All the assays were performed at 20 °C.

2.6. Protein assay

Protein concentration was determined according to Bradford [20].

2.7. Mass spectrometry

Elution of proteins from acrylamide gels, trypsin digestion, and separation of peptides were performed as previously described [21]. The resulting peptides were analyzed by LC-MS/MS using an Orbitrap XL instrument (Thermo Fisher, Waltham, MA, USA) equipped with a nano-ESI source coupled with a nano-Acquity capillary UPLC (Waters, Milford, MA, USA). Briefly, peptides were separated with a capillary BEH C18 column (0.075 × 100 mm, 1.7 μM, Waters) using aqueous 0.1% formic acid (A) and CH_3CN containing 0.1% formic acid (B) as mobile phases. Peptides were eluted by means of a linear gradient from 5% to 50% of B in 90 min, at a 300 nL/min flow rate. Mass spectra were acquired over an *m/z* range from 400 to 1800. To achieve protein identification, MS and MS/MS data underwent Mascot Search Engine software analysis to interrogate the National Center for Biotechnology Information nonredundant (NCBInr) protein database. Parameters sets were: trypsin cleavage; carbamidomethylation of cysteines as a fixed modification and methionine oxidation as a variable modification; a maximum of two missed cleavages; false discovery rate, calculated by searching the decoy database, 0.05.

3. Results

3.1. Steady-state assays with crude enzyme

As a first test, we assayed the activity of crude β-glucosidase towards *p*-nitrophenyl-β-D-glucopyranoside (pNPGluc) as a function of pH. To this aim, we dissolved the lyophilized enzyme powder in a universal buffer (composed of MES, MOPS, and Tris, 25 mM each) equilibrated at pH 5.5, 6, 6.25, 6.5, 6.75, 7, or 7.5. The enzyme aliquots accordingly prepared were then used to assay the activity, at each pH value considered, as a function of pNPGluc concentration (Fig. 1A and B). The apparent K_m and V_{max} were determined, and the decrease of