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A Pseudo-β-glucosidase in *Arabidopsis thaliana*: Correction by Site-Directed Mutagenesis, Heterologous Expression, Purification, and Characterization

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Abstract—Since *At2g25630* is an intronless gene with a premature stop codon, its cDNA encoding the predicted mature β-glucosidase isoenzyme was synthesized from the previously isolated *Arabidopsis thaliana* genomic DNA. The stop codon was converted to a sense codon by site-directed mutagenesis. The native and mutated cDNA sequences were separately cloned into the vector pPICZαB and expressed in *Pichia pastoris*. Only the cells transformed with mutated cDNA-vector construct produced the active protein. The mutated recombinant β-glucosidase isoenzyme was chromatographically purified to apparent homogeneity. The molecular mass of the protein is estimated as ca. 60 kD by SDS-PAGE. The pH optimum of activity is 5.6, and it is fairly stable in the pH range of 5.0-8.5. The purified recombinant β-glucosidase is effectively active on *para*-/*ortho*-nitrophenyl-β-D-glucopyranosides (*p*-/*o*-NPG) and 4-methylumbelliferyl-β-D-glucopyranoside (4-MUG) with K_m values of 1.9, 2.1, 0.78 mM and k_{cat} values of 114, 106, 327 nkat/mg, respectively. It also exhibits different levels of activity against *para*-/*ortho*-nitrophenyl-β-D-fucopyranosides (*p*-/*o*-NPF), amygdalin, prunasin, cellobiose, gentiobiose, and salicin. The enzyme is competitively inhibited by gluconolactone and *p*-nitrophenyl-1-thio-β-D-glucopyranoside with *p*-NPG, *o*-NPG, and 4-MUG as substrates. The enzyme is found to be very tolerant to glucose inhibition. The catalytic role of nucleophilic glutamic acid in the motif YITENG of β-glucosidases and mutated recombinant enzyme is discussed.

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Key words: *Arabidopsis thaliana*, β-glucosidase, site-directed mutagenesis, heterologous expression, *Pichia pastoris*

β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) selectively catalyzes the hydrolytic cleavage of β-glycosidic bond between two or more glycone residues or of that between glucose and an aryl or alkyl aglycone. The enzyme constitutes a major group among glycoside hydrolases that occur universally in all living organisms from bacteria to humans. β-Glucosidases play key roles in a variety of essential physiological processes and potential biotechnological applications depending on the nature and diversity of the glycone or aglycone moiety of their substrates. Among the mammalian β-glucosidases, the human acid β-glucosidase, commonly known as glucocerebrosidase, catalyzes the degradation of glucosylceramide in the lysosome. The deficiency of the enzyme leads to an inherited Gaucher's disease [1]. β-Glucosidases in cellulolytic microorganisms have recently been the focus of much research since cellulose is the most abundant substrate on earth and is very likely to be an important renewable resource of energy in the future [2-4]. Plant β-glucosidases have been reported to be involved in regulation of the physiological activity of phytohormones by hydrolysis of their inactive hormone-glucoside conjugates [5, 6], chemical defense against pests [7-9], lignification [10], β-glucan synthesis during cell wall development and cell wall degradation in the endosperm during germination [11, 12], and food quality and flavor enhancement [13].

The completion of the *Arabidopsis* genome sequencing project enabled researchers to determine also the putative β-glucoside glucohydrolases. *At2g25630* is an intronless gene of *A. thaliana*, apparently encoding an

Abbreviations: 4-MUG) 4-methylumbelliferyl-β-D-glucopyranoside; *p*-/*o*-NPF) *para*-/*ortho*-nitrophenyl-β-D-fucopyranosides; *p*-/*o*-NPG) *para*-/*ortho*-nitrophenyl-β-D-glucopyranosides.

inactive β-glucosidase isoenzyme due to a premature stop codon. The present paper describes for first time the activation, purification, and characterization of the inactive recombinant β-glucosidase isoenzyme expressed heterologously in the yeast *Pichia pastoris* following conversion of the premature stop codon to a sense codon by site-directed mutagenesis.

MATERIALS AND METHODS

DNA isolation, site-directed mutagenesis, and construction of plasmids. Genomic DNA was isolated from 3-week-old *A. thaliana* seedlings using whole plant parts [14]. Since the related gene (*At2g25630*) is intronless, the cDNA encoding the predicted mature β-glucosidase isoenzyme was synthesized from the genomic DNA by PCR using the high fidelity Pfu^{TM} Turbo DNA polymerase (Stratagene, USA) with the sense primer 5′-CACTCTGCAGCACCTAAATTAAGAAAAACTGATTTC-3′ and the antisense primer 5′-TATTGGCGGCCGCCATATCTCATCAATTCTCCTTTTTTCC-3′, introducing *Pst*I and *Not*I restriction sites (underlined), respectively. Following the purification from gel with the QiaQuick gel extraction kit (Qiagen, Germany), the predicted premature stop codon was converted to a sense codon by using the gel pure native cDNA as template and mutated complementary sense oligonucleotide 5′-GCATCAGATTGGCTTTTGATATATC-3′ and antisense oligonucleotide 5′-GATATATCAAAAGCCAATCTGATGC-3′ as primers in combination with a 5′- or a 3′-end gene specific primer in PCR and then fusing the resulting PCR products by overlap extension [15]. The native and mutated PCR products encoding the native and mutated β-glucosidase isoenzymes, respectively, were separately digested with *Pst*I and *Not*I restriction enzymes, and then each insert (native and mutated cDNAs) was gel-purified and cloned into the *P. pastoris* expression vector pPICZαB (Invitrogen, USA) that had been double-digested with *Pst*I and *Not*I in advance. This vector allows expression of a cloned cDNA under the control of methanol-inducible alcohol oxidase gene (*AOX*1) and secretion of the recombinant protein into the culture medium. The native signal peptide sequences of both native and mutated cDNAs were analyzed using the SignalP v.2.0 program and replaced with the α-signal sequence of the vector, targeting the protein to the secretory pathway. The DNA sequence analysis was established by using BigDye terminator technology (REFGEN Biyoteknoloji, Turkey).

Transformation and selection of productive transformants. The wild-type *P. pastoris* strain X33 was separately transformed with *Pme*I-linearized pPICZαB-native cDNA and pPICZαB-mutated cDNA constructs using the chemical method. Transformants were selected for their ability to grow on zeocin-agar plates according to the manufacturer's instructions (Invitrogen). Small-scale expression trials were performed to identify transformants with the highest β-glucosidase activity and to define the optimal expression conditions. Protein expression and secretion was monitored at 0, 12, 24, 48, 72, and 96 h time points by enzyme activity assay.

Large-scale expression and purification of recombinant β-glucosidase. Two hundred milliliters of buffered glycerol-complex medium (BMGY) containing 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 0.34% yeast nitrogen base, 1% ammonium sulfate, $4 \cdot 10^{-5}$ % biotin, and 1% glycerol was inoculated with 20 ml preculture of a *P. pastoris* transformant and grown until the culture reached an A_{600} ca. 6 in a rotary shaking incubator at 30°C and 250 rpm. The cells were harvested by centrifuging at 3000*g* for 5 min at room temperature. The cell pellet was resuspended in BMM (buffered minimal methanol) medium (100 mM potassium phosphate, pH 6.0, 0.34% yeast nitrogen base, 1% ammonium sulfate, $4 \cdot 10^{-5}$ % biotin, and 1% methanol) using 2× of the original culture volume and returned to the shaking incubator at 30°C and 180 rpm for expression. Methanol (100%) was added to a final concentration of 1% every 24 h to maintain induction. The culture supernatant containing recombinant β-glucosidase was obtained after 72 h by centrifugation for 20 min at 4°C and 12,000*g* and used as crude extract for further purification.

All purification steps were performed at 4°C unless otherwise stated. The crude enzymic extract was treated with solid ammonium sulfate to obtain the 20-75% fraction after centrifuging at 15,000*g* for 1 h. The precipitate was dissolved in 25 mM potassium phosphate buffer, pH 6.0. The enzyme solution was loaded onto a Sephacryl S-300 HR column (1.5 × 80 cm) pre-equilibrated and eluted with 25 mM potassium phosphate buffer, pH 6.0, at room temperature. The flow rate was 20 ml/h, and 1.5 ml fractions were collected. Fractions with β-glucosidase activity were pooled and then applied on a DEAE-Sepharose ion-exchange column (1 × 14 cm) pre-equilibrated with 25 mM potassium phosphate buffer, pH 6.0. The enzyme was eluted with a linear gradient of 0-1 M KCl in the same buffer at a flow rate of 30 ml/h, and 1 ml fractions were collected. Fractions showing the highest activity were pooled and dialyzed overnight against 50 mM sodium acetate buffer, pH 5.6. The desalted enzyme preparation was applied to a CM-Sepharose ion-exchange column (1 × 14 cm) pre-equilibrated with 50 mM sodium acetate buffer, pH 5.6, and the effluent was collected in 1 ml fractions. The enzyme was eluted with a linear gradient of 0-1 M KCl in the same buffer at a flow rate of 30 ml/h, and 1 ml fractions were collected.

The protein having the highest β-glucosidase activity, mostly present in the unbound fractions, was combined and dialyzed overnight against 25 mM potassium phosphate buffer, pH 6.0. The desalted enzyme preparation was applied again to the DEAE-Sepharose ion-

exchange column and chromatographed as above. Fractions showing the highest activity were pooled and concentrated by ultrafiltration (Amicon Ultra-15; Millipore, USA). The concentrated enzyme solution was used as purified β-glucosidase for subsequent studies after confirming homogeneity by gel electrophoresis.

β-Glucosidase assays and protein determinations. During enzyme extraction and purification, recombinant β-glucosidase activity was routinely determined using *para*- and *ortho*-nitrophenyl-β-D-glucopyranosides (*p*-NPG and *o*-NPG) as substrates. Appropriately diluted 70 μl of enzyme solution and 70 μl of substrate were mixed in the wells of a 96-well microtiter plate in quadruplicate. After incubation at 37°C for 30 min, the reaction was stopped by adding 70 μl of 0.5 M Na_2CO_3, and the color that developed as a result of *p*-/*o*-nitrophenol liberation was measured at 410 nm. Enzyme activity was expressed as nmol *p*-nitrophenol formed per second (nkat) in the reaction mixture under these assay conditions. When the substrate used did not contain *p*-/*o*-nitrophenol, the recombinant enzyme activity was determined by the coupled glucose oxidase/peroxidase assay procedure (Sigma, USA). Zymogram assays were carried out for detection of recombinant β-glucosidase activity after native PAGE under non-denaturing conditions using 4-methylumbelliferyl-β-D-glucopyranoside (4-MUG) as a substrate.

Protein determination. Protein concentrations were determined according to Bradford [16] using bovine serum albumin (BSA) as a standard.

Polyacrylamide gel electrophoresis (PAGE). For SDS-PAGE, protein samples were fractionated on 12% SDS-polyacrylamide gels [17] using a Minigel system (Thermo Scientific, USA). Gels were fixed, stained with Coomassie brilliant blue R-250 (Sigma), and destained using standard methods to detect protein bands. When detection of recombinant β-glucosidase activity was required in a non-denaturing electrophoresis process, the enzyme solutions were loaded onto 6% native polyacrylamide gel. After electrophoresis, the gel was equilibrated in two changes of 50 mM sodium acetate buffer, pH 5.6, for 15 min each, and then incubated with the substrate 4-MUG for 15 min at 37°C. The released methylumbelliferone was observed and photographed under UV light.

Determination of pH optimum and stability. The effect of varying pH values on recombinant β-glucosidase activity and stability was determined using 25 mM sodium acetate, citrate-phosphate, phosphate, and glycine-NaOH buffers for the pH ranges of 3.0-5.8, 3.0-7.0, 6.0-8.5, and 8.5-10.5, respectively. For determining the profile of pH stability, samples of the recombinant enzyme solution were incubated at 37°C for 2 h. The solutions were diluted 5 times with 200 mM sodium acetate buffer, pH 5.6 (optimum for the recombinant enzyme), and assayed for the residual activity using 5 mM *p*-NPG as substrate in 200 mM sodium acetate buffer, pH 5.6.

Determination of temperature effect on the enzyme reaction and denaturation. For temperature optimum determination, the recombinant enzyme and substrate *p*-NPG solution mixtures were incubated in the temperature range 4-80°C for 30 min, and the residual activity was measured. For measuring thermostability, the recombinant enzyme was first incubated at different temperatures (4-80°C) in 50 mM sodium acetate buffer, pH 5.6, in the absence of substrate for 10 min. The activity was subsequently assayed at 37°C as described above.

Kinetic parameters. Various final concentrations of *p*-NPG (0.78-20 mM), *o*-NPG (0.78-20 mM), and 4-MUG (0.078-2 mM) were used to estimate the kinetic parameters K_m and k_{cat}. Inhibition experiments were performed using *p*-NPG, *o*-NPG, and 4-MUG as substrates at concentrations of 1 to 10 K_m, and in different final concentrations of gluconolactone (0.39-25 mM), *p*-nitrophenyl 1-thio-β-D-glucopyranoside (0.31-10 mM), and glucose (0.5-250 mM) as inhibitors. The double reciprocal Lineweaver–Burk plot was used to calculate the parameters.

RESULTS AND DISCUSSION

Sequence alignment data of *A. thaliana* At2g25630 revealed very high similarity with protein sequences of an *A. thaliana* putative β-glucosidase isoenzyme (93.7%), *Prunus serotina* prunasin hydrolase-I (71%), *P. serotina* amygdalin hydrolase-I (69.2%), *Zea mays* β-glucosidase-I (59.4%), and *Sorghum bicolor* dhurrinase-I (65.4%). The primary protein structure deduced from At2g25630 contains the strictly conserved peptide motifs SAYQ, YRFSI, TLNEP, APGRCS, GINYY, YITENG, and DNFEW, which serve as fingerprints to identify an unknown protein as a member of family 1 β-glucosidase (Fig. 1). TLNEP and YITENG are the most conserved ones to make up a part of the active site and contain the two key catalytic glutamic acids [18, 19]. Substrate hydrolysis of β-glucosidases has been reported to involve enzyme glycosylation and deglycosylation steps and requires participation of the nucleophilic glutamic acid and acid/base catalyst glutamic acid residues in the motifs YITENG and TLNEP, respectively [20, 21]. However, the premature translation termination was determined (X-385) in the sequence of At2g25630 instead of W found in the analyzed glucoside hydrolases (Fig. 1). Since the nucleophilic glutamic acid (E-413), located in the motif YITENG, is at the C-terminal side (downstream) of the premature stop codon, the *At2g25630* gene of *A. thaliana* should encode an inactive β-glucosidase isoenzyme. To clarify this prediction and further confirm the catalytic role of nucleophilic glutamic acid in the motif YITENG of β-glucosidases, the native (the cDNA with premature stop codon and its protein will be referred to as native hereafter) and subsequently mutated cDNAs of *A.*

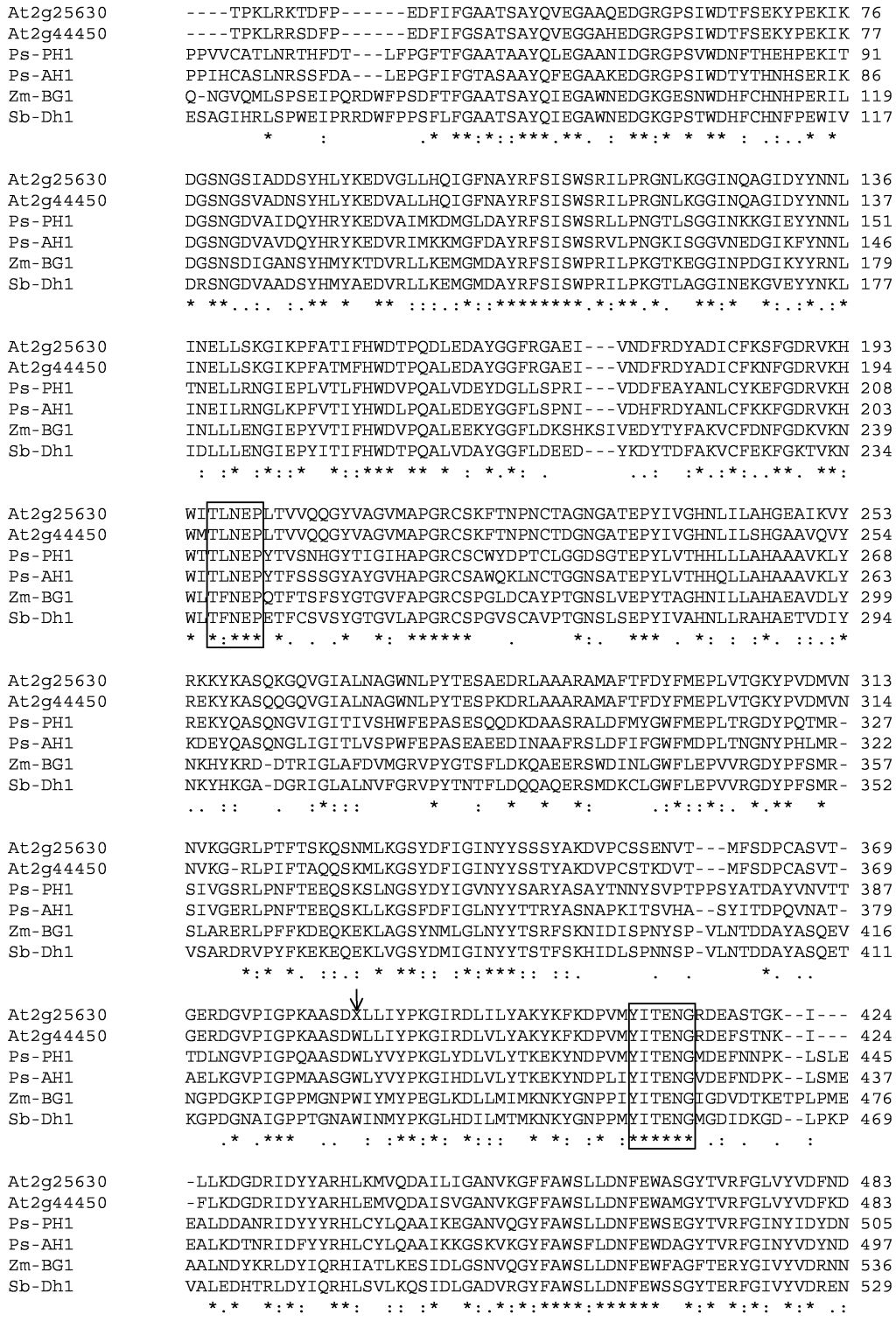

Fig. 1. Sequence alignment of *A. thaliana* β-glucosidase isoenzyme (At2g25630; GenBank accession AC006053), *A. thaliana* putative β-glucosidase isoenzyme (At2g44450; GenBank accession AC004521), *Prunus serotina* prunasin hydrolase-I (Ps-PH1; GenBank accession U50201), *P. serotina* amygdalin hydrolase-I (Ps-AH1; GenBank accession U26025-AF411130), *Zea mays* β-glucosidase-I (Zm-BG1; GenBank accession U25157), and *Sorghum bicolor* dhurrinase-I (Sb-Dh1; GenBank accession U33817). The arrow indicates the premature stop position of At2g25630. The two boxed peptide motifs TF/LNEP and YITENG are highly conserved in family 1 β-glucosidases. They contain two key catalytic glutamic acids and also form the glycone-binding site within the active site. The symbols denoting the degree of conservation observed in each column are as follows: "*" means that the residues in that column are identical in all sequences in the alignment; ":" means that a conserved substitution is observed; "." means that a semi-conserved substitution is observed; dashes indicate gaps (deletions) that the alignment software (Clustal W, 1.82) introduced to optimize the alignment.

thaliana At2g25630 were separately cloned for heterologous expression in the yeast *P. pastoris*.

Because *At2g25630* is an intronless gene, which was probably derived by retrotransposition after reverse transcription of the mature mRNA transcript of a progenitor β-glucosidase gene, its cDNA encoding the predicted mature β-glucosidase isoenzyme was synthesized from the previously isolated *A. thaliana* genomic DNA. The cDNA was mutated replacing adenine nucleotide by guanine nucleotide with directed mutagenesis to convert the predicted premature stop codon (TGA) to a sense codon (TGG) encoding Trp amino acid (Fig. 2). The native and mutated cDNA sequences were separately cloned into the *P. pastoris* expression vector pPICZαB. The native signal peptide sequences of both native and mutated cDNAs were replaced by the α-signal peptide of the vector to ensure protein secretion into the culture medium through the secretory pathway. *Pichia pastoris* transformants, separately containing pPICZαB-native cDNA and pPICZαB-mutated cDNA constructs were screened for β-glucosidase activity, and only the ones transformed with mutated cDNA were found to produce the active protein, as predicted from the sequence analysis data. Transformants with the highest recombinant enzyme activity were chosen to optimize and upscale protein expression.

The culture supernatant having the highest level of secreted recombinant β-glucosidase activity in 72 h of expression was obtained by centrifugation and used as crude enzymic extract for further purification. Upon fractionation of the β-glucosidase active fractions with ammonium sulfate, 96% of the activity was obtained in the fraction saturated with 20-75% ammonium sulfate. After gel filtration chromatography on a Sephacryl S-300 HR column, the enzyme was found in fractions 75-110 and pooled. Anion-exchange chromatography of the combined active fraction on a DEAE-Sepharose column removed the greater part of the contaminants and decreased total protein amount from 9.3 to 0.43 mg. When the eluted active fractions from DEAE-Sepharose column were pooled and applied to a CM-Sepharose column, most of the contaminants bound while β-glucosidase did not, retaining 88% of the activity from the previous step. The effluent containing β-glucosidase activity

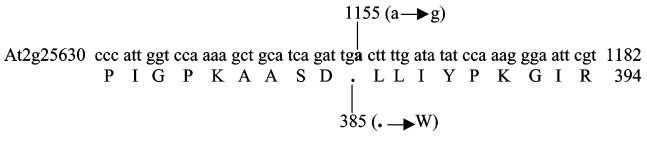

Fig. 2. A selected region of cDNA and amino acid sequences of *A. thaliana* β-glucosidase isoenzyme. The premature stop codon TGA due to a single nucleotide substitution at the 1155th position is shown. Adenine nucleotide was replaced by guanine nucleotide with directed mutagenesis to convert the sense codon encoding Trp at the 385th position.

Table 1. Purification of the mutated recombinant *A. thaliana* β-glucosidase isoenzyme expressed in *P. pastoris*

Step	Total protein, mg	Specific activity, nkat/mg	Yield, %
Culture supernatant	27.6	16.3	100.0
Ammonium sulfate	15.2	28.5	96
Sephacryl S-300 HR	9.3	39.8	82
DEAE-Sepharose	0.43	138	13.2
CM-Sepharose	0.22	237	11.5
DEAE-Sepharose	0.09	339	6.8

was applied again on DEAE-Sepharose column to remove the remaining contaminants. The recombinant enzyme was purified 21-fold to homogeneity with an overall enzyme yield of 6.8% and a specific activity of 339 nkat/mg protein (Table 1).

SDS-PAGE analysis exhibited that only the "corrected" form of the cDNA by directed mutagenesis gave rise to a recombinant protein band of ca. 60 kD, which is slightly higher than the calculated mass of At2g25630 β-glucosidase (Fig. 3a, lane *4*). The difference between this value and the theoretical molecular mass of 54 kD presumably reflects the carbohydrate side-chains of the recombinant protein. The cDNA with a predicted premature stop codon was also heterologously expressed and a polypeptide with a smaller size (ca. 40 kD) compared with the mutated protein was produced (Fig. 3a, lane *3*). However, there was no detectable β-glucosidase protein band from the expression culture medium of *P. pastoris* transformed with empty vector pPICZαB as negative control (Fig. 3a, lane *2*). The molecular mass of the recombinant mutated β-glucosidase, 60 kD, is similar to those for many other plant β-glucosidases, which generally exhibit values in the range of 50-65 kD [12, 22].

In order to confirm the activity data of recombinant native and mutated proteins with spectrophotometric assays, native PAGE zymogram assay was performed. The zymogram profile was developed on gel that yielded a zone of recombinant β-glucosidase activity of identical electrophoretic mobility for mutated protein with the fluorescent substrate 4-MUG (Fig. 3b, lane *3*). Negative control medium and recombinant native cDNA expression product did not yield an activity zone with 4-MUG, as predicted (Fig. 3b, lanes *1* and *2*, respectively).

The pH optimum for recombinant mutated β-glucosidase activity was 5.6 (Fig. 4), and the enzyme retained over 50% of the original activity between pH 4.0 and 7.5. This pH optimum is in agreement with the pre-

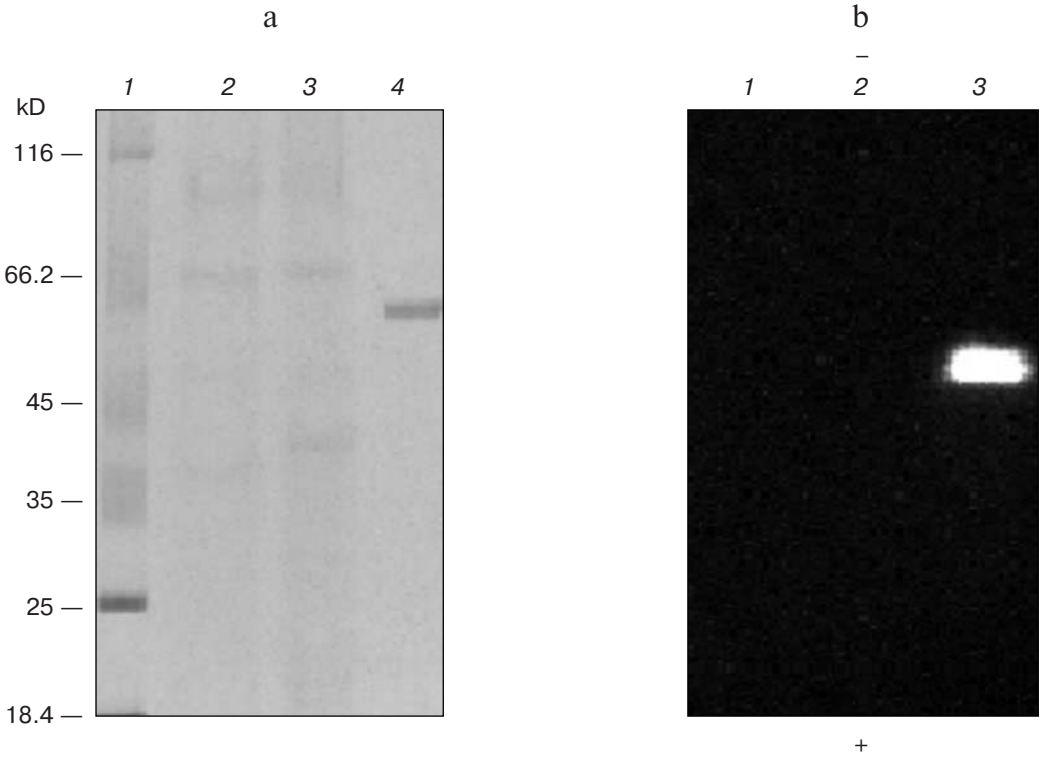

Fig. 3. a) SDS-PAGE of native and mutated recombinant β-glucosidases expressed in *P. pastoris*. The proteins were electrophoresed at pH 8.3 on a 12% acrylamide gel and stained with Coomassie brilliant blue R-250. Lanes: *1*) molecular weight standards (Fermentas Life Sciences, Lithuania) (β-galactosidase, 116 kD; bovine serum albumin, 66.2 kD; ovalbumin, 45 kD; lactate dehydrogenase, 35 kD; REase Bsp981, 25 kD; β-lactoglobulin, 18.4 kD); *2*) expression medium supernatant of *P. pastoris* host transformed with empty vector; *3*) medium supernatant of *P. pastoris* expressing native recombinant β-glucosidase; *4*) mutated recombinant pure β-glucosidase. b) Native PAGE (6%) gel zymogram of recombinant β-glucosidases developed with the fluorogenic substrate 4-MUG. Lanes: *1*) expression medium supernatant of *P. pastoris* host transformed with empty vector; *2*) medium supernatant of *P. pastoris* expressing native recombinant β-glucosidase; *3*) mutated recombinant β-glucosidase.

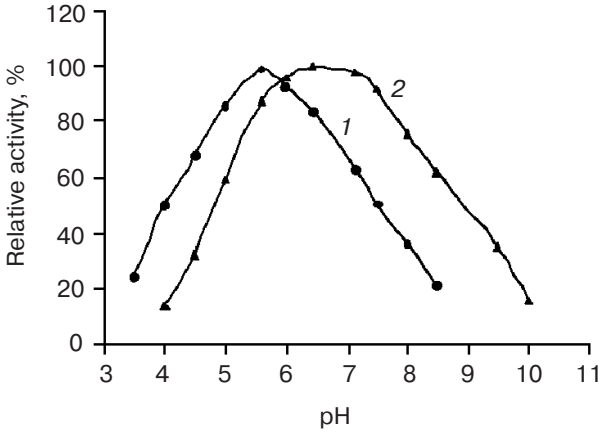

Fig. 4. Effect of pH on activity (*1*) and stability (*2*) of purified recombinant *A. thaliana* (mutated) β-glucosidase isoenzyme expressed in *P. pastoris*.

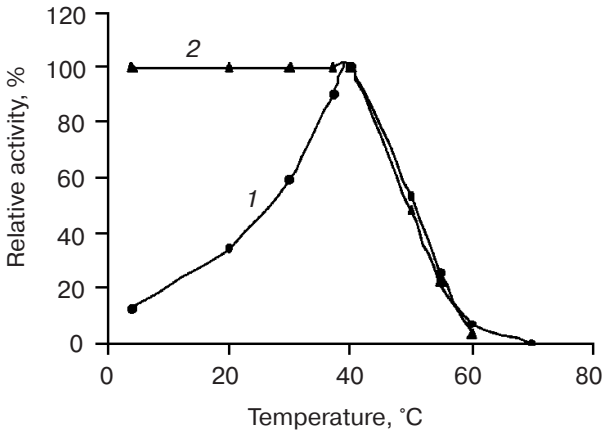

Fig. 5. Effect of temperature on activity (*1*) and stability (*2*) of purified recombinant *A. thaliana* (mutated) β-glucosidase isoenzyme expressed in *P. pastoris*.

viously determined optimum pH values of β-glucosidases from various plant sources, between 4.0 and 7.0 [22-25]. The recombinant mutated β-glucosidase was very stable between pH values 6.0 and 7.2, retaining over 98% activity after incubation at 37°C for 2 h, and also fairly stable at pH 5.0 and 8.5, exhibiting 60 and 62% activities, respectively, under the same conditions (Fig. 4). The enzyme displayed maximal activity at 40°C (Fig. 5),

which is lower than for most plant β-glucosidases that show the highest enzymatic activity at 50°C [22, 25, 26], although some have a similar temperature optimum [27].

Increased catalytic activity at higher temperatures, such as 50°C, is not physiologically meaningful because the activity is lost due to thermal denaturation in a very short incubation time. Thermostability of the enzyme at different temperatures was monitored by measuring its activity at 37°C. The enzyme was fairly stable in 50 mM sodium acetate buffer, pH 5.6, at temperatures up to 42°C, while retaining only 48% of the original activity at 50°C for 10 min. It was completely inactivated upon incubation at 60°C for 10 min (Fig. 5). This property is common for mesophilic β-glucosidases, which are irreversibly inactivated at and above 55-60°C.

The substrate specificity of the recombinant mutated β-glucosidase was determined towards various artificial and natural substrates. The enzyme exhibited different levels of activity against alkyl-glucopyranosides, most aryl-glucopyranosides, and other β-linked disaccharides (Table 2). It was effectively active on *p*-NPG, *o*-NPG, and 4-MUG with relative activities of 1, 0.95, and 1.63, respectively. Rates of hydrolysis of *para*- and *ortho*-nitrophenyl-β-D-fucopyranosides (*p*-/*o*-NPF) were 0.7 and 1.16, respectively, relative to *p*-NPG. Similarly, higher activity rates of β-glucosidases from vanilla bean, butter bean, and wheat seedlings for *p*-/*o*-NPF have been reported [27-29], however, these fucopyranosides competitively inhibited β-glucosidase activity from the yeast *P. pastoris* against *p*-NPG, *o*-NPG, and 4-MUG as substrates [30]. Relatively high and similar activity was observed for naturally occurring cyanogenic alkyl-β-glucopyranosides prunasin and amygdalin (0.39 and 0.48, respectively, relative to *p*-NPG). However, quite different hydrolysis rates of these cyanogenic substrates by plant β-glucosidases have been reported. For instance, vanilla bean β-glucosidase hydrolyses prunasin three times higher than *p*-NPG, while it has no activity against amygdalin [27]. Also prunasin was effectively hydrolyzed by butter bean and ripe sweet cherry fruit β-glucosidases [28, 31]. None or negligibly low recombinant mutated β-glucosidase activity was determined towards other possible substrates investigated (Table 2).

The reaction kinetics of the purified recombinant mutated β-glucosidase were determined from Lineweaver–Burk plots with *p*-NPG, *o*-NPG, and 4-MUG as substrates (Table 3). The K_m value for *p*-NPG

Table 2. Relative activity of the mutated recombinant *A. thaliana* β-glucosidase isoenzyme expressed in *P. pastoris*

Substrate	Relative activity, %
p-Nitrophenyl β-D-glucopyranoside (*p*-NPG)	100
p-Nitrophenyl 1-thio-β-D-glucopyranoside	0
p-Nitrophenyl β-D-fucopyranoside (*p*-NPF)	70.6
p-Nitrophenyl β-D-mannopyranoside	0
p-Nitrophenyl β-D-galactopyranoside	0
o-Nitrophenyl β-D-glucopyranoside (*o*-NPG)	95
o-Nitrophenyl β-D-galactopyranoside	17.2
o-Nitrophenyl β-D-fucopyranoside (*o*-NPF)	117
4-Methylumbelliferyl β-D-glucopyranoside (4-MUG)	163
n-Octyl-β-D-glucopyranoside	6.9
n-Decyl-β-D-glucopyranoside	10.6
n-Heptyl-β-D-glucopyranoside	16.5
D(+)Cellobiose	6.3
β-Gentiobiose	6.3
Amygdalin	48
Prunasin	39
Arbutin	0
Salicin	4.4

Note: Purified β-glucosidase was incubated at its optimum pH (pH 5.6) with potential substrates provided at 10 mM final concentrations. Enzyme activity was determined by measuring the rate of *p*-NPG/*o*-NPG production at 410 nm with subsequent use of respective standard curves. For the substrates that do not contain *p*-/*o*-nitrophenol, the enzyme activity was determined by the coupled glucose oxidase/peroxidase assay procedure. Reaction rates are expressed here as a percentage of that observed with *p*-NPG.

Table 3. Kinetic parameters of the mutated recombinant *A. thaliana* β-glucosidase isoenzyme expressed in *P. pastoris*

Substrate	K_m, mM	k_{cat}, nkat/mg
p-NPG	1.9	114
o-NPG	2.1	106
4-MUG	0.78	327

Table 4. Competitive inhibition of the mutated recombinant *A. thaliana* β-glucosidase isoenzyme expressed in *P. pastoris*

Substrate	K_i, mM	
	gluconolactone	*p*-nitrophenyl 1-thio-β-D-glucopyranoside
p-NPG	2.0	4.5
o-NPG	2.2	6.9
4-MUG	4.7	2.2

was similar to those reported for vanilla bean [27] and wheat seedlings [29] β-glucosidases.

The inhibition experiments of the recombinant mutated enzyme were performed using *p*-NPG, *o*-NPG, and 4-MUG as substrates and gluconolactone, *p*-nitrophenyl 1-thio-β-D-glucopyranoside, and glucose as inhibitors. Gluconolactone was the most effective competitive inhibitor of the enzymatic activity with K_i values of 2.0 and 2.2 mM against *p*-NPG and *o*-NPG, respectively. The K_i value for this inhibitor was twice higher (4.7 mM) towards the substrate 4-MUG. This strongly inhibitory effect of gluconolactone is in agreement with the previous reports regarding the inhibition of β-glucosidases from various plant sources [10, 27, 32]. Contrary to gluconolactone, the *p*-nitrophenyl 1-thio-β-D-glucopyranoside was the most effective inhibitor of the enzyme with the K_i value of 2.2 mM, when 4-MUG used as the substrate. The inhibition constant values for the latter inhibitor were 4.5 and 6.9 mM against *p*-NPG and *o*-NPG, respectively (Table 4). Similar to various plant and microorganism β-glucosidases [27, 33, 34], the recombinant mutated β-glucosidase from *A. thaliana* was not inhibited by glucose up to 250 mM.

In conclusion, the present study has revealed the heterologous expression of both an *A. thaliana* pseudo β-glucosidase gene with a predicted premature stop codon and of the cDNA corrected by directed mutagenesis to produce catalytically active protein for the first time. The recombinant isoenzyme has been compared with reported plant β-glucosidases following purification to homogeneity. Moreover, the absolute necessity of the nucleophilic glutamic acid residue, located in the motif YITENG, for the substrate hydrolysis of β-glucosidase has been confirmed.

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