Biochimica et Biophysica Acta 1850 (2015) 1877–1883

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Substrate specificities of two tau class glutathione transferases inducible by 2,4,6-trinitrotoluene in poplar

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ARTICLE INFO

Article history:
Received 14 February 2015
Received in revised form 30 April 2015
Accepted 20 May 2015
Available online 27 May 2015

Keywords:
Glutathione transferase
Detoxication
Populus trichocarpa
Phytoremediation
TNT

ABSTRACT

Background: The genome of poplar (*Populus trichocarpa*) encodes 81 glutathione transferases (GSTs) annotated in eight distinct classes. The tau class is considered the most versatile in the biotransformation of xenobiotics and is composed of 58 GSTs. Two of the enzymes, GSTU16 and GSTU45, have particular interest since their expression is induced by exposure of poplar tissues to 2,4,6-trinitrotoluene (TNT) and could potentially be involved in the metabolism of this toxic environmental contaminant.
Results: DNA encoding these GSTs was synthesized and the proteins were heterologously expressed in *Escherichia coli* and the purified enzymes were characterized.
Major conclusions: GSTU16 assayed with a number of conventional GST substrates showed the highest specific activity (60 μmol min^{-1} mg^{-1}) with phenethyl isothiocyanate, 150-fold higher than that with CDNB. By contrast, GSTU45 showed CDNB as the most active substrate (3.3 μmol min^{-1} mg^{-1}) whereas all of the 16 alternative substrates tested yielded significantly lower activities. Homology modeling suggested that the aromatic residues Phe10 and Tyr107 in the active site of GSTU16 are promoting the high activity with PEITC and other substrates with aromatic side-chains. Nonetheless, TNT was a poor substrate for GSTU16 as well as for GSTU45 with a specific activity of 0.05 nmol min^{-1} mg^{-1} for both enzymes.
General significance: GSTU16 and GSTU45 do not play a major role in the degradation of TNT in poplar.

1. Introduction

Glutathione transferases (GSTs; EC 2.5.1.18) are known as a superfamily of phase II detoxication enzymes occurring in different forms in most organisms from mammalians to plants and even in many prokaryotes. The enzymes catalyze the reaction of reduced glutathione (GSH) with electrophilic groups of endogenous or xenobiotic compounds and facilitate elimination of toxins by producing more water-soluble products [1].

Based on structure and other properties the numerous GSTs can be divided into different classes [2]. In plants the tau class (GSTU) is the most numerous featuring 58 members in poplar (*Populus trichocarpa*), 28 in *Arabidopsis thaliana* and 20 in *Zea mays* [3–5]. The enzymes are involved in protection of cells subjected to biotic and abiotic stress by catalyzing the conjugation and inactivation of variety of herbicides, heavy metals and other toxic compounds [6–8]. Apart from their catalytic functions GSTs are also involved in development and growth of cells in response to red light [9].

A major societal concern is the contamination of the environment by man-made chemical compounds, and it has been proposed that phytoremediation by means of plants endowed with suitable biochemical functions could be effective in the cleanup of polluted soil [10]. For efficient phytoremediation fast-growing poplar have been identified as suitable plants for the treatment of large areas in a cost-effective and environmentally friendly manner [11]. GSTs have been considered as useful for the inactivation of certain herbicides and explosive materials such as 2,4,6-trinitrotoluene (TNT) and 1,3,5-trinitro-1,3,5-triazine (RDX) [12,13] and a pertinent question is whether the enzyme activity in poplar is potent enough for the intended purpose.

In this study two members of the tau class in *Populus trichocarpa*, GSTU16 and GSTU45, were characterized. Previously, known as GST173 and GST180, these enzymes are both induced in root tissues of hydroponic poplar plantlets treated with TNT and of potential relevance to phytoremediation of this explosive [14]. Coding sequences of DNA optimized for heterologous expression of GSTU16 and GSTU45 in *Escherichia coli* were synthesized and the purified enzymes were characterized and compared by means of kinetic studies with a variety of substrates and the use of modeled structures.

Abbreviations: Allyl-ITC, allyl isothiocyanate; Benzyl-ITC, benzyl isothiocyanate, CDNB, 1-chloro-2,4-dinitrobenzene; CuOOH, cumene hydroperoxide; DHA, dehydroascorbate; DNT, 2,4-dinitrotoluene; GST, glutathione transferase; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; PEITC, phenethyl isothiocyanate; Propyl-ITC, propyl isothiocyanate; TNT, 2,4,6-trinitrotoluene

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http://dx.doi.org/10.1016/j.bbagen.2015.05.015
0304-4165/

2. Methods

2.1. Expression and purification of poplar GSTU16 and GSTU45

Poplar GSTU16 (ADB11318) and GSTU45 (ADB11327) DNA sequences in the pJ401 expression vector were obtained from DNA 2.0 Inc. (Menlo Park, CA, USA). Without changes of the encoded amino acid sequences, both sequences were codon-optimized for expression in *E. coli* and were also supplied with an N-terminal His_6-tag (Supplementary Figs. 1 and 2). *E. coli* BL21 Gold (DE3) competent cells were transformed by heat shocking at 42 °C and grown overnight on agar plates containing kanamycin (50 μg/ml). A single colony was picked and inoculated into 50 ml LB medium (10 g tryptone, 5 g yeast extract, 10 g NaCl and 50 μg/ml kanamycin) and grown overnight in a shaking incubator at 37 °C, and 5 ml culture was then transferred into 500 ml 2TY (16 g tryptone, 10 g yeast extract, 5 g NaCl including 50 μg/ml kanamycin) medium. Expression of proteins was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when the absorbance of the culture at 600 nm was 0.3–0.6. The culture was further incubated for 6 h and cell pellets were obtained by centrifugation at 1500 *g* for 10 min. The pellets were kept at −80 °C until the purification step.

The pellets were dissolved in ice-cold lysis buffer (50 ml washing buffer containing one tablet of protease inhibitor cocktail, 0.2 mg/ml lysozyme) with gently mixing on ice for 30 min and then disrupted further by using a sonicator (5× for 20 s). Clear lysate was obtained by centrifugation at 15,000 *g*, 4 °C for 1 h.

His GraviTrap affinity columns (GE Healthcare) pre-packed with Ni-Sepharose were used for purification of His-tagged GSTU16 and GSTU45 proteins. The purification was following the procedure in the manufacturer's protocol. Briefly, columns were equilibrated with washing/binding buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4) prior to use. Following adsoption of protein lysates, columns were washed thoroughly with washing/binding buffer. GSTU16 was eluted with 40 mM and GSTU45 was eluted with 100 mM imidazole in binding buffer, respectively. Proteins were dialyzed (10 mM Tris HCl pH 7.8, 1 mM EDTA, 0.2 mM DTT) overnight and finally the purity of the proteins was checked with SDS-PAGE.

2.2. Measurement of GST activities

GST activities were measured with seventeen different substrates using a Shimadzu UV-2501 PC spectrophotometer with a thermostated cell compartment adjusted to 30 °C. Absorbance change was measured in quartz cuvettes for 1 min and the pH values of the buffers were chosen differently for alternative substrates to minimize the non-enzymatic reaction rates. Activities were calculated from the initial slopes of reactions and molar extinction coefficients of substrates at selected wavelengths.

The principles for assays with the alternative substrates have previously been published as follows: for 1-chloro-2,4-dinitrobenzene (CDNB) [15]; for 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) [16]; for phenethyl isothiocyanate (PEITC), allyl isothiocyanate (Allyl-ITC), benzyl isothiocyanate (Benzyl-ITC), propyl isothiocyanate (Propyl-ITC), and sulforaphane [17]; for iodomethane, iodobutane and iodooctane [18]; for cumene hydroperoxide (CuOOH), hydrogen peroxide (H_2O_2), and dehydroascorbate (DHA) [19]; for trans-2-hexenal and trans-2-Nonenal [20]; for TNT and DNT [13] as indicated in Supplementary Table 1.

2.3. Kinetic parameters

CDNB, PEITC, Allyl-ITC, sulforaphane and CuOOH substrates were chosen for further steady-state kinetic analysis. Measurements were made under the same conditions as used for specific activity measurements using at least six different concentrations of substrate and a fixed concentration of GSH. V_{max} and K_m values were obtained by non-linear regression analysis using GraphPad Prism software, and k_{cat} values were calculated based on the mass of the dimer, known as the functional molecular form. In the experiments CDNB was used in the range of 0.025 to 2 mM with 5 mM GSH, isothiocyanates (PEITC, allyl-ITC, sulforaphane) were used in the range of 0.0125 to 1 mM with 1 mM GSH, and CuOOH was used in the range of 0.025 to 6 mM with 1 mM GSH. In all assays the content ethanol and acetonitrile used to dissolve substrates were 5% (v/v) or 0.5% (v/v), respectively, concentrations without any inhibitory effect on enzyme activity. All measurements were performed at least in triplicate.

2.4. Sequence alignment and structure modeling

The crystal structures 2VO4 and 4J2F obtained from PDB were used for modeling the GSTU16 and GSTU45 proteins, respectively. The amino acid sequences of the proteins were aligned with Clustal Omega [21].

Chimera software was used for modeling of the 3D structures of proteins and analysis of the modeled protein structures [22]. The GSTU16 and GSTU45 models were matched with the 2VO4 template, which contained S-(p-nitrobenzyl)glutathione and possible interactions of modeled proteins with the ligand in the active sites were determined.

3. Results

3.1. Expression and purification of poplar GSTU16 and GSTU45

GSTU16 and GSTU45 were expressed in *E. coli* BL21 (DE3) cells at 30 °C and purified with His GraviTrap Ni-Sepharose small columns with a total yield of 15 and 8 mg proteins for GSTU16 and GSTU45, respectively. Both enzymes were obtained in homogeneous form as

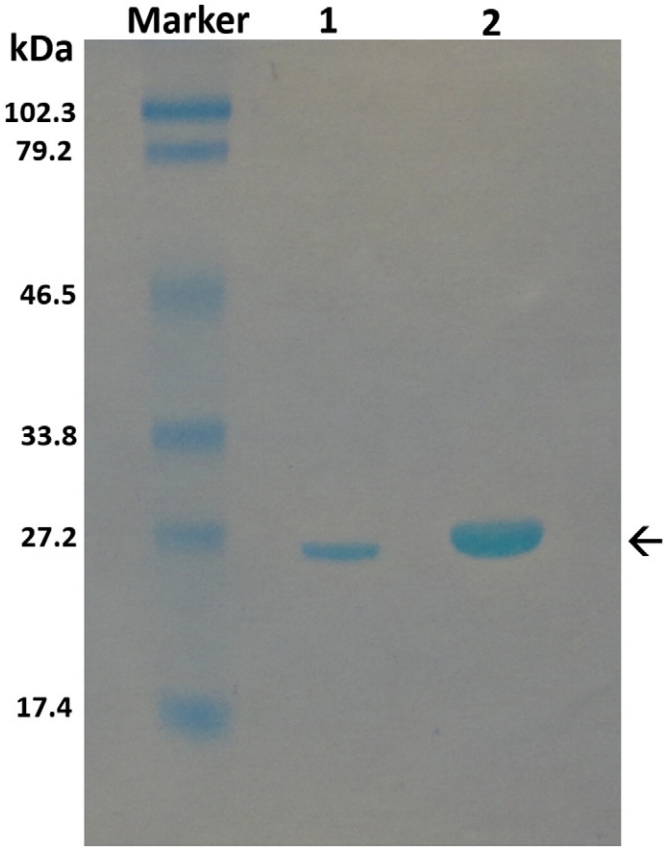

Fig. 1. SDS-PAGE analysis of purified poplar GSTs. Markers, GSTU16 (lane 1) and GSTU45 (lane 2) are shown with an arrow indicating 25 kDa.