Biochemistry and Biophysics Reports 5 (2016) 141–145

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

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

Drosophila GSTs display outstanding catalytic efficiencies with the environmental pollutants 2,4,6-trinitrotoluene and 2,4-dinitrotoluene

Aslam M.A. Mazari, Bengt Mannervik*

Department of Neurochemistry, Stockholm University, SE-10691 Stockholm, Sweden

ARTICLE INFO

Article history:
Received 29 October 2015
Received in revised form
20 November 2015
Accepted 1 December 2015
Available online 4 December 2015

Keywords:
Glutathione conjugation
2,4,6-trinitrotoluene detoxication
2,4-dinitrotoluene detoxication
Drosophila GSTs
Human GSTs

ABSTRACT

The nitroaromatic explosive 2,4,6-trinitrotoluene (TNT) and the related 2,4-dinitrotoluene (DNT) are toxic environmental pollutants. The biotransformation and detoxication of these persistent compounds in higher organisms are of great significance from a health perspective as well as for the biotechnological challenge of bioremediation of contaminated soil. We demonstrate that different human glutathione transferases (GSTs) and GSTs from the fruit fly *Drosophila melanogaster* are catalysts of the biotransformation of TNT and DNT. The human GSTs had significant but modest catalytic activities with both DNT and TNT. However, *D. melanogaster* GSTE6 and GSTE7 displayed outstanding high activities with both substrates.

1. Introduction

Glutathione transferases (GSTs; EC 2.5.1.18) belong to a family of abundant phase II detoxication enzymes involved in the metabolism and inactivation of a broad range of structurally unrelated endogenous metabolites and xenobiotic electrophiles through glutathione (GSH) conjugation. Thereby, cells are provided with necessary protection against various cytotoxins [1–3].

GSTs have been found in almost all aerobic organisms from insects to plants to mammals, and even in many prokaryotes. On the basis of their amino acid sequences and structural similarities, the numerous soluble mammalian GSTs (also known as canonical or cytosolic GSTs) can be divided into seven different classes designated by their Greek names, alpha, mu, omega, pi, sigma, theta and zeta [4]. In insects six classes of soluble GSTs have been identified. The fruit fly *Drosophila melanogaster* contains omega, sigma, theta and zeta classes of GSTs, which appear to exist in almost all eukaryotes, plus two additional classes, delta and epsilon. The epsilon class is the most numerous class of soluble GSTs in *Drosophila melanogaster* represented by 14 GST genes [5].

GSTs exhibit broad substrate specificities towards various endogenous and xenobiotic electrophiles including aryl halides, α,β-unsaturated carbonyls, oxidized lipids, isothiocyanates, various drugs and pollutants [1,3,6,7]. Although many of these reactions are catalyzed by several different GSTs, each GST isoform shows its own substrate selectivity [2,8]. The nitroaromatic explosive 2,4,6-trinitrotoluene (TNT) and its precursor 2,4-dinitrotoluene (DNT) are important occupational and environmental pollutants introduced into nature by human activities. Many nitro-substituted explosives have been evaluated in laboratory studies and found to be toxic for almost all classes of organisms including algae, bacteria, plants and mammals [9].

Two plant tau class GSTs from *Arabidopsis thaliana* [10] as well as from poplar (*Populus trichocarpa)* along with an equine GST have been reported to have catalytic activities with TNT *in vitro* [11,12]. However no data have yet been reported showing human and insect GSTs catalyzing the conjugation of GSH with TNT and the closely related DNT (Fig. 1).

In the present investigation we have determined the catalytic activities with TNT and DNT displayed by a set of purified GSTs. Seven human GSTs from four different classes, namely, GSTA1-1 and GSTA2-2 (alpha class), GSTM2-2, GSTM4-4, and GSTM5-5 (mu class), GSTP1-1 (pi class), GSTS1-1(sigma class), plus two GSTs from the *Drosophila melanogaster* epsilon class, DmGSTE6 and DmGSTE7. Further kinetic studies of the most active enzymes DmGSTE6 and DmGSTE7 were performed with both substrates.

2. Materials and methods

2.1. Materials

Unless stated otherwise, all chemicals used for enzymatic activity and kinetic measurements were purchased from

* Corresponding author.
E-mail address: Bengt.Mannervik@neurochem.su.se (B. Mannervik).

http://dx.doi.org/10.1016/j.bbrep.2015.12.003

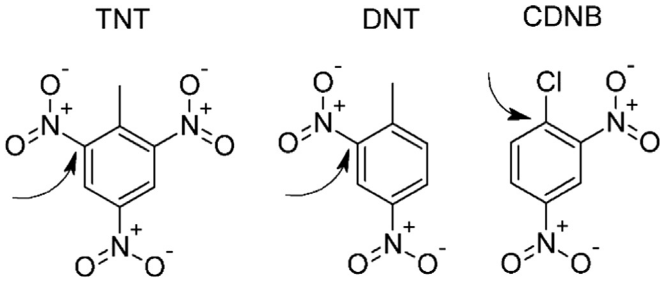

Fig. 1. Structural similarities between CDNB, DNT and TNT. The arrow head shows the site of attack by GSH.

Sigma-Aldrich. TNT was kindly provided by Dr. Rune Berglind, Swedish Defence Research Agency (FOI).

2.2. *Expression and purification of recombinant GSTs*

The genes encoding DmGST6, DmGSTE7 and human GSTS1-1 were custom synthesized by DNA 2.0 (Menlo Park, CA, USA) and were provided in the pJexpress 401 expression vector with N-terminal His_6-tags. *Escherichia coli* XL1-Blue electrocompetent cells were transformed by the electroporation technique and the bacteria were grown overnight on LB-agar plates containing 50 μg/ml kanamycin. A starter culture of 50 ml LB-medium containing the appropriate antibiotic was inoculated with a single colony and the cells were allowed to grow at 37 °C at 200 rpm in an incubator. After 16 h a larger culture of 500 ml LB-medium was inoculated with 5 ml of starter culture. The GST expression was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside at an optical density of $OD_{600\ nm} \approx 0.4$. The bacteria were further allowed to grow at 37 °C for 16 h and cell pellets were obtained by centrifugation at 7000 rpm for 10 min at 4 °C. The supernatant was discarded and the pellets were kept at −80 °C until the purification was performed by Ni-IMAC as described previously [13]. Briefly, the pellets were dissolved in 25 ml of ice-cold buffer A (20 mM sodium phosphate buffer pH 7.8, supplemented with 85 mM imidazole, 500 mM NaCl, 10 mM β-mercaptoethanol, 0.02% NaN_3) and 0.2 mg/ml lysozyme, half a tablet of EDTA-free protease inhibitor (Roche Germany) and incubated for 30 min on an ice bath. The resultant suspension was lysed by sonication 5 × 20 s and centrifuged at 27,200*g* for 45 min at 4 °C. The cell debris was discarded and the supernatant containing the enzyme was incubated with pre-equilibrated Ni-IMAC gel on an ice bath for 30 min. The gel was packed into a column and the unbound proteins were washed away with buffer A. The bound enzyme was eluted with 500 mM imidazole (otherwise identical with buffer A) at a flow rate of 1 ml/min. The eluted fractions were pooled and dialyzed overnight against 10 mM Tris HCl buffer pH 7.8, containing 0.2 mM DTT and 1 mM EDTA. The other human GST isoenzymes were heterologously expressed in *E.coli* and purified by GSH-affinity chromatography as described by Kolm *et al.* [14]. The protein concentrations of the recombinant enzymes were determined by the Bradford assay [15] and the purity of the enzymes was assessed by SDS-PAGE. In order to verify that the purified GSTs were active, the standard GST substrate 1-chloro-2,4-dinitrobenzene (CDNB) was used.

2.3. *Enzyme activity assay*

The enzymatic activities of the purified GSTs with 1 mM DNT or 0.2 mM TNT with 1 mM GSH were determined in 0.1 M sodium phosphate buffer, pH 6.5, at 30 °C for 30 min. The stock solutions of DNT were prepared in ethanol (resulting in 5% final ethanol concentration in 1 ml reaction mixture), while TNT stock was provided as 0.5 mM aqueous solution. After 30 min incubation the nitrite formation was assayed colorimetrically as described by French *et al.* [16] by using the Griess assay with modifications as follows. To a 360 μl of reaction sample, 360 μl of milli-Q H_2O and 180 μl of sulfanilamide (10 mg/ml in 0.68 M HCl) were added. The components were mixed well and incubated at room temperature for 10 min. After addition of 72 μl of *N*-(1-naphthyl)ethylenediamine (10 mg/ml in H_2O), and mixing and further incubation for 10 min the absorbance was measured spectrophotometrically at 540 nm. The nitrite released from the nitroaromatic compounds was quantified from a sodium nitrite standard curve with known concentrations. The blank reactions were prepared by using the same concentrations of the substrates without enzymes and the values were subtracted from the enzymatic reactions.

2.4. *Kinetic measurements*

Steady-state kinetic analyses of DmGSTE6 and DmGSTE7 with DNT and TNT substrates were performed with 3–13 μg enzyme in the assay under the same conditions as for the specific activity determinations. For the determination of kinetic parameter values, saturation curves were obtained by using at least seven different concentrations of DNT and TNT with a saturating GSH concentration of 5 mM. The reactions were linear for at least 30 min; $< 6\%$ of TNT and $< 0.3\%$ of DNT were consumed in that time under the conditions used. Nitrite formation was determined after 30 min by the Griess assay as described earlier. The TNT concentrations used were between 0.0125 and 0.4 mM, while DNT concentrations were varied from 0.025 to 5 mM. The concentrations of the TNT and DNT stock solutions limited the experiments to nonsaturating substrate levels.

2.5. *Data analysis*

All reactions for determination of both specific activities and the steady-state kinetic parameters were performed in triplicate. The nitrite formation for each reaction was quantified from a standard curve with known concentrations. The kinetic parameters values were obtained from GraphPad Prism 6.0 software by using non-linear regression analysis and the Michaelis–Menten equation. The k_{cat} and k_{cat}/K_m values were calculated from the subunit concentrations of the enzymes used for the reactions.

3. Results

3.1. *DNT and TNT as substrates for GSTs*

To investigate the environmental pollutants and toxicants DNT and TNT as substrates for GSTs, a set of GSTs from the human alpha, mu, pi, and sigma classes as well as two epsilon class GSTs from *D. melanogaster* was used. Table 1 shows the catalytic activities of nine different enzymes with DNT and TNT measured under standard assay conditions in 0.1 M sodium phosphate buffer pH 6.5 at 30 °C. The temperature was chosen for comparison with previously published GST activities with other substrates at 30 °C. The DNT and TNT concentrations were below the corresponding K_m values and substrate saturation could not be obtained owing to limited solubility of the substrates in the assay system. Among the tested enzymes, the human GSTs were the least active with both substrates as compared to *D. melanogaster* DmGSTE6 and DmGSTE7, which showed higher specific activities. DmGSTE6 was the most active enzyme with TNT displaying a specific activity of 62.7 ± 2.6 nmol min^{-1} mg^{-1}. DmGSTE7 showed a specific activity of 20.0 ± 2.0 nmol min^{-1} mg^{-1}, which is 3-fold lower than that of DmGSTE6. However, the specific activities of DmGSTE6

(20.5 ± 1.4 nmol min^{-1} mg^{-1}) and DmGSTE7 (14.3 ± 1.4 nmol min^{-1} mg^{-1}) with DNT were more similar in magnitude. Among the different human GSTs, GSTA2-2 demonstrated the highest specific activity with DNT (2.6 ± 0.4 nmol min^{-1} mg^{-1}), whereas < 0.01 nmol min^{-1} mg^{-1} activity was detected for this enzyme with TNT. By contrast, GSTM4-4 showed minute activity with TNT (0.09 ± 0.01 nmol min^{-1} mg^{-1}) but < 0.01 nmol min^{-1} mg^{-1} activity with DNT. Other human GSTs showed comparatively moderate specific activities with both substrates. GSTS1-1 showed the lowest specific activities with both DNT and TNT (Table 1).

Table 1
Specific activities of GSTs from different sources with TNT and DNT as substrates. The results are the means of 3 replicate measurements ± S.E. The background reactions without enzyme were measured by using the same concentration of the solvent and subtracted from the rates in the presence of enzyme.

Enzyme	Specific activity (nmol min^{-1} mg^{-1})	
	TNT	DNT
DmGSTE6	62.7 ± 2.6	20.5 ± 1.4
DmGSTE7	20.0 ± 2.0	14.3 ± 1.4
GSTP1-1	0.7 ± 0.1	0.6 ± 0.1
GSTA1-1	0.3 ± 0.1	0.9 ± 0.2
GSTA2-2	< 0.01	2.6 ± 0.4
GSTM2-2	0.3 ± 0.01	0.8 ± 0.1
GSTM4-4	0.09 ± 0.01	< 0.01
GSTM5-5	2.1 ± 0.1	1.1 ± 0.2
GSTS1-1	0.01 ± 0.003	0.07 ± 0.01
GSTU16 (Poplar)[a]	0.055 ± 0.012	0.42 ± 0.05
GSTU45 (Poplar)[a]	0.050 ± 0.010	0.048 ± 0.004
GSTU24 (Arabidopsis)[b]	0.67	Not tested
GSTU25 (Arabidopsis)[b]	1.0	Not tested

[a] Data from [16].
[b] Estimated from Fig. 2.8. in Vanda Gunning M.Sc. thesis, University of York, UK.

3.2. Steady-state kinetic parameters

Among all the tested enzymes DmGSTE6 and DmGSTE7 showed high specific activities with both DNT and TNT, and these enzymes were further characterized by determining their steady-state kinetic parameters. All the measurements were carried out at a saturating GSH concentration of 5 mM and by varying the concentrations of DNT and TNT (Fig. 2). Although rate saturation could not reached in the range of experimentally accessible substrate concentrations, the precision of the measurements allowed determination of the kinetic parameters by nonlinear regression analysis. The results summarized in Table 2 show that the catalytic efficiency (k_{cat}/K_m) of DmGSTE6 with TNT is almost 3-fold higher than that of DmGSTE7, while the k_{cat} values were not significantly different. With DNT as substrate both the k_{cat} and K_m values were approximately two-fold higher for DmGSTE6 than for DmGSTE7. As a result, the catalytic efficiencies of both the enzymes were similar.

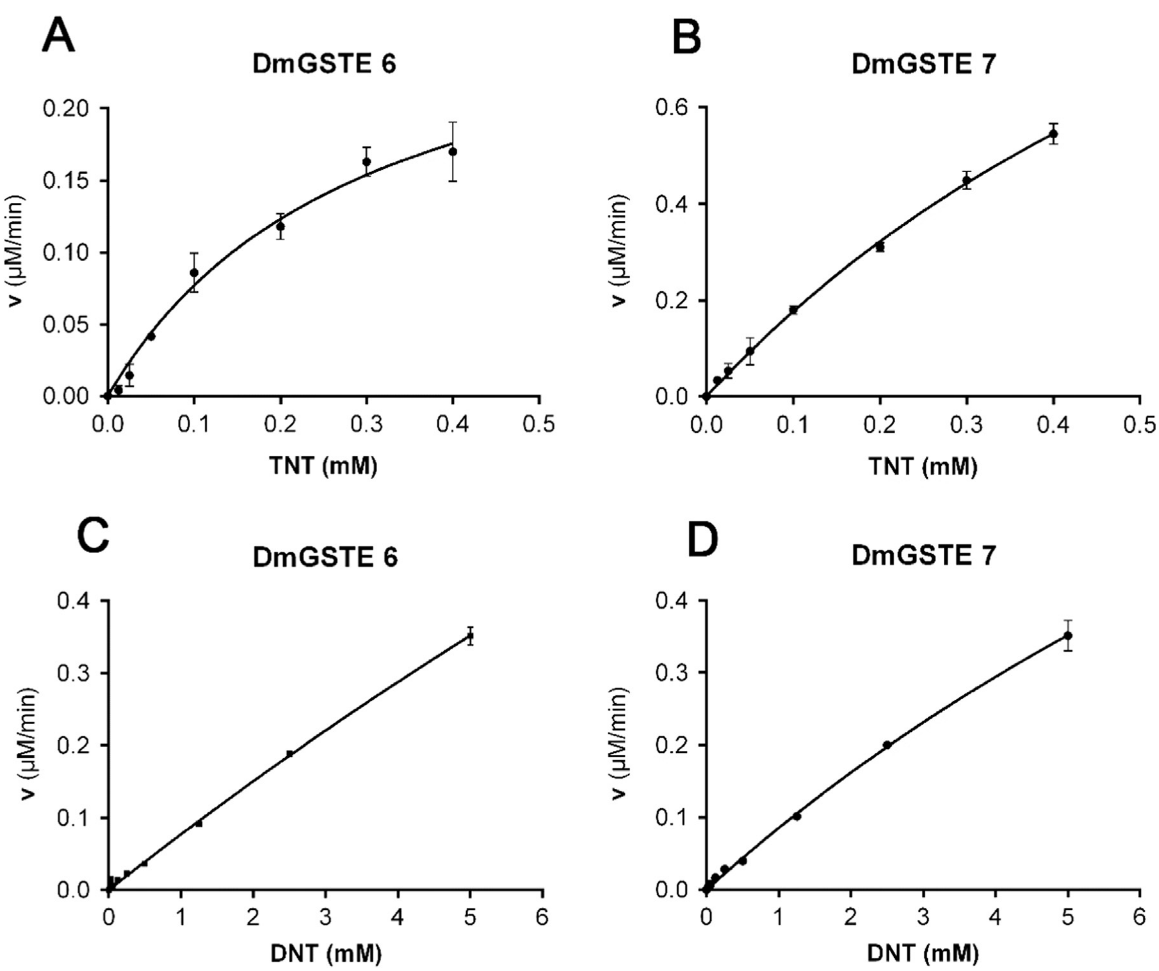

Fig. 2. Saturation curves of DmGSTE6 and DmGSTE7 with DNT and TNT.The reactions were performed in triplicate in the presence of a saturating GSH concentration of 5 mM and by varying the concentrations of electrophilic substrate TNT (A, B) from 0.0125 to 0.4 mM and DNT (C, D) 0.025 to 5 mM for half an hour. The nitrite formation was monitored by using the Griess assay and the results were quantified by using a standard nitrite curve. The non-enzymatic reactions were performed in the same manner without enzyme and the curves have been adjusted to account for non-enzymatic background reactions. The data were fitted by non-linear regression using the program GraphPad Prism 6.0. Each point in the graph represents the average of three individual replicate measurements with mean ± S.E.

Table 2
Steady-state kinetic parameters of DMGSTE6 and DmGSTE7 with TNT and DNT as substrates. The reactions were performed in triplicate in the presence of 5 mM fixed GSH concentration and by varying the concentrations of the electrophilic substrates DNT and TNT. The nitrite formation was assayed by using the Griess assay and the results were quantified by using a standard nitrite curve. The non-enzymatic reactions were performed in the same manner without enzymes.

Substrate	DmGSTE6	DmGSTE7
TNT		
K_m (mM)	0.30 ± 0.06	0.91 ± 0.15
k_{cat} (s^{-1})	0.05 ± 0.005	0.06 ± 0.008
k_{cat}/K_m (mM^{-1} s^{-1})	0.172 ± 0.039	0.062 ± 0.014
DNT		
K_m (mM)	40.78 ± 17.61	17.78 ± 3.51
k_{cat} (s^{-1})	0.74 ± 0.29	0.35 ± 0.06
k_{cat}/K_m (mM^{-1} s^{-1})	0.018 ± 0.011	0.019 ± 0.005

4. Discussion

Living organisms are exposed to a large number of non-nutritional chemical species, referred to as xenobiotics. The interaction of such chemicals with living organisms can have deleterious effects, often causing severe toxic as well as carcinogenic effects [17]. Advances in the chemical industry during the past decades have greatly increased the number of man-made chemicals such as pharmaceuticals, pesticides, insecticides and other environmental pollutants, which in turn pose an ever increasing challenge to living organisms. To limit the threat posed by endogenously produced toxic compounds as well as the man-made chemical species, organisms have evolved complex detoxication systems fundamental to their survival [18].

DNT and TNT are nitroaromatic compounds that derive from ammunition. The release of these nitro-substituted explosives in the soil and surface waters due to military and industrial activities generates significant health risks to humans as well as to the ecological system. Due to their chemically resistant structures, a number of nitroaromatic compounds including DNT and TNT are highly recalcitrant to degradation and are mineralized at a slow pace by microorganisms. TNT is relatively more refractory to biodegradation than mono- and dinitrotoluenes due to its symmetric distribution of the nitro groups on the aromatic ring, limiting the attack of microbial enzymes that are fundamental in the biodegradation of aromatic compounds in the environment [19,20]. TNT is considered a C-class human carcinogen and its metabolites have been shown to exhibit variable levels of cytotoxicity and mutagenicity in bacterial and mammalian cell systems [21,22]. Occupational exposure of TNT has also been associated with various clinical manifestations including aplastic anaemia, dermatitis and toxic hepatitis [23,24]. For the above stated reasons, the biotransformation of TNT has been extensively studied worldwide, and various enzymes from plants and microorganisms have been reported to be involved in the metabolism of TNT [19,25]. A recent study of *Arabidopsis thaliana* clarified that monodehydroascorbate reductase 6 plays a pivotal role in TNT toxicity by generating a nitroradical that promotes formation of reactive oxygen species [26]. However detoxication of TNT and related compounds in mammals and insects remains largely unexplored.

GSTs constitute large group of phase II detoxication enzymes evolved via multiple gene duplications to diverse classes of enzymes that fulfill a broad spectrum of functional roles including detoxication of xenobiotics. GSTs act by catalyzing the reaction of the tripeptide GSH with numerous different chemical structures, suggesting that GSTs might play an important role in the biotransformation of TNT and its metabolites [11,27]. Previously, a few tau class GSTs from poplar and *Arabidopsis* have been shown to have catalytic activities with TNT [10,12]. Two of the poplar GSTs were reported to be up-regulated in response to TNT [11,27,28]. However, the observed catalytic activities of these tau class GSTs appears not to be efficient enough to play an important role in the biotransformation of TNT.

In order to identify catalytically efficient GST enzymes that could efficiently catalyze the biotransformation of DNT and TNT, we have subjected a set of human GSTs along with two epsilon class GSTs from *D. melanogaster* to activity studies. The results summarized in Table 1 demonstrate that DmGSTE6 and DmGSTE7 have higher catalytic activities with both substrates compared to the human GSTs and the plant enzymes previously studied [10,12]. Among the tested enzymes, DmGSTE6 was characterized as the most efficient enzyme with a specific activity of 62.7 ± 2.6 nmol min^{-1} mg^{-1}, which is >1000 times more active than the previously reported poplar GSTU16 and GSTU45 [12]. DmGSTE7 was the second best enzyme with a specific activity of 20.0 ± 2.0 nmol min^{-1} mg^{-1}, 3-fold lower than that of DmGSTE6. The catalytic activities of DmGSTE6 and DmGSTE7 with DNT as substrate were somewhat lower and of similar magnitude.

Fig. 1 shows that TNT and DNT have structural similarities to 1-chloro-2,4-dinitrobenzene (CDNB), known as a "universal" substrate for GSTs [29]. However, CDNB is chemically substantially more reactive than TNT and DNT as shown by the second-order rate constants (k_2) of the nonenzymatic reactions. At pH 6.5 and 30 °C, k_2 for CDNB is $16{,}000 \pm 950$ nM^{-1} s^{-1} as compared to 10 ± 0.9 and 1.9 ± 0.2 nM^{-1} s^{-1} for TNT and DNT, respectively. The chloride ion is more readily displaced from CDNB than is nitrite from TNT and DNT. Our assay monitors the release of nitrite and the consequent formation of a glutathione conjugate is evidenced by thin layer chromatography (unpublished data), but the site of conjugation requires further studies of the products. The specific activities of DmGSTE6 and DmGSTE7 with CDNB are approximately 1000-fold higher than with TNT and DNT, 74,000 and 36,000 nmol min^{-1} mg^{-1}, respectively. A pertinent question is therefore if genetic engineering of the *Drosophila* GSTs could significantly improve the TNT and DNT activities. Enhanced activities by three orders of magnitude have previously been accomplished by rational redesign of several GSTs [30–32]. Similarly, improving the activity of plant GSTs by genetic engineering could possibly find applications for the phytoremediation of contaminated environments. Uptake of TNT and conjugation with glutathione has been shown to lead to plant resistance and detoxication of the pollutant [10].

Crystal structures of DmGSTE6 and DmGSTE7 have recently been determined [33]. Both enzymes belong to the epsilon class of GSTs, which is not represented in mammals or plants, but the structures do not reveal any features that could explain the high TNT and DNT activities. The chain-folds of soluble mammalian, plant, and insect GSTs are highly similar and the CDNB activities of DmGSTE6 and DmGSTE7 are similar in magnitude to most mammalian GSTs. Therefore, the exact topography of the active site accommodating the electrophilic substrate obviously governs the substrate selectivity of the different enzymes.

In conclusion, the identification of two *Drosophila* enzymes with distinguishing high catalytic activities with TNT and DNT underline the potential of genetic engineering of GSTs for applications in biotransformation and phytoremediation, as evidenced by the overexpression of GSTs in *A. thaliana* [10].

Acknowledgments

We thank Professor Neil C. Bruce, University of York, UK for valuable advice on the assay of TNT activity and Dr. Rune Berglind, Swedish Defence Research Agency (FOI) for providing TNT stock solutions. In our laboratory Dr. Birgitta Sjödin and Dr. Yaman Musdal have provided GST preparations and valuable advice and Mr. Aram Ismail verified formation of a glutathione conjugate in the reaction. This study has been supported by grants from the Swedish Research Council (Grant no. 2012-5161).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.12.003.

References

[1] P.D. Josephy, B. Mannervik, Molecular Toxicology, 2nd ed., Oxford University Press, New York (2006), p. 589.
[2] R.N. Armstrong, Structure, catalytic mechanism, and evolution of the glutathione transferases, Chem. Res. Toxicol. 10 (1997) 2–18.
[3] J.D. Hayes, J.U. Flanagan, I.R. Jowsey, Glutathione transferases, Annu. Rev. Pharmacol. Toxicol. 45 (2005) 51–88.
[4] B. Mannervik, P.G. Board, J.D. Hayes, I. Listowsky, W.R. Pearson, Nomenclature for mammalian soluble glutathione transferases, Methods Enzymol. 401 (2005) 1–8.
[5] C. Saisawang, J. Wongsantichon, A.J. Ketterman, A preliminary characterization of the cytosolic glutathione transferase proteome from Drosophila melanogaster, Biochem. J. 442 (2012) 181–190.
[6] A.A. Enayati, H. Ranson, J. Hemingway, Insect glutathione transferases and insecticide resistance, Insect. Mol. Biol. 14 (2005) 3–8.
[7] B. Mannervik, U.H. Danielson, Glutathione transferases – structure and catalytic activity, CRC Crit. Rev. Biochem. 23 (1988) 283–337.
[8] A.J. Oakley, Glutathione transferases: new functions, Curr. Opin. Struct. Biol. 15 (2005) 716–723.
[9] B. Van Aken, Transgenic plants for enhanced phytoremediation of toxic explosives, Curr. Opin. Biotechnol. 20 (2009) 231–236.
[10] V. Gunning, K. Tzafestas, H. Sparrow, E.J. Johnston, A.S. Brentnall, J.R. Potts, E. L. Rylott, N.C. Bruce, Arabidopsis glutathione transferases U24 and U25 exhibit a range of detoxification activities with the environmental pollutant and explosive, 2,4,6-trinitrotoluene, Plant Physiol. 165 (2014) 854–865.
[11] L.B. Brentner, S.T. Mukherji, K.M. Merchie, J.M. Yoon, J.L. Schnoor, B. Van Aken, Expression of glutathione S-transferases in poplar trees (Populus trichocarpa) exposed to 2,4,6-trinitrotoluene (TNT), Chemosphere 73 (2008) 657–662.
[12] Y. Musdal, B. Mannervik, Substrate specificities of two tau class glutathione transferases inducible by 2,4,6-trinitrotoluene in poplar, Biochim. Biophys. Acta. 1850 (2015) 1877–1883.
[13] A.M. Mazari, O. Dahlberg, B. Mannervik, M. Mannervik, Overexpression of glutathione transferase E7 in Drosophila differentially impacts toxicity of organic isothiocyanates in males and females, PLoS One 9 (2014) e110103.
[14] R.H. Kolm, G. Stenberg, M. Widersten, B. Mannervik, High-level bacterial expression of human glutathione transferase P1-1 encoded by semisynthetic DNA, Protein. Expr. Purif. 6 (1995) 265–271.
[15] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.
[16] C.E. French, S. Nicklin, N.C. Bruce, Aerobic degradation of 2,4,6-trinitrotoluene by Enterobacter cloacae PB2 and by pentaerythritol tetranitrate reductase, Appl. Environ. Microbiol. 64 (1998) 2864–2868.
[17] B.N. Ames, M. Profet, L.S. Gold, Nature's chemicals and synthetic chemicals: comparative toxicology, Proc. Natl. Acad. Sci. USA 87 (1990) 7782–7786.
[18] D. Sheehan, G. Meade, V.M. Foley, C.A. Dowd, Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily, Biochem. J. 360 (2001) 1–16.
[19] A. Esteve-Nunez, A. Caballero, J.L. Ramos, Biological degradation of 2,4,6-trinitrotoluene, Microbiol. Mol. Biol. Rev. 65 (2001) 335–352.
[20] N.K. Hannink, S.J. Rosser, N.C. Bruce, Phytoremediation of explosives, Crit. Rev. Plant Sci. 21 (2002) 511–538.
[21] M.E. Honeycutt, A.S. Jarvis, V.A. McFarland, Cytotoxicity and mutagenicity of 2,4,6-trinitrotoluene and its metabolites, Ecotoxicol. Environ. Saf. 35 (1996) 282–287.
[22] B. Lachance, P.Y. Robidoux, J. Hawari, G. Ampleman, S. Thiboutot, G.I. Sunahara, Cytotoxic and genotoxic effects of energetic compounds on bacterial and mammalian cells in vitro, Mutat. Res. 444 (1999) 25–39.
[23] D. Rosenblatt, E. Burrows, W. Mitchell, D. Parmer, Organic explosives and related compounds, in: O. Hutzinger (Ed.), The Handbook of Environmental Chemistry-Anthropogenic Compounds, Springer Verlag, Berlin, 1991, pp. 195–237.
[24] D.R. Lima, M.L. Bezerra, E.B. Neves, F.R. Moreira, Impact of ammunition and military explosives on human health and the environment, Rev. Environ. Health 26 (2011) 101–110.
[25] N. Hannink, S.J. Rosser, C.E. French, A. Basran, J.A. Murray, S. Nicklin, N. C. Bruce, Phytodetoxification of TNT by transgenic plants expressing a bacterial nitroreductase, Nat. Biotechnol. 19 (2001) 1168–1172.
[26] E.J. Johnston, E.L. Rylott, E. Beynon, A. Lorenz, V. Chechik, N.C. Bruce, Monodehydroascorbate reductase mediates TNT toxicity in plants, Science 349 (2015) 1072–1075.
[27] M.P. Mezzari, K. Walters, M. Jelinkova, M.C. Shih, C.L. Just, J.L. Schnoor, Gene expression and microscopic analysis of Arabidopsis exposed to chloroacetanilide herbicides and explosive compounds. A phytoremediation approach, Plant Physiol. 138 (2005) 858–869.
[28] D.R. Ekman, W.W. Lorenz, A.E. Przybyla, N.L. Wolfe, J.F. Dean, SAGE analysis of transcriptome responses in Arabidopsis roots exposed to 2,4,6-trinitrotoluene, Plant Physiol. 133 (2003) 1397–1406.
[29] A.G. Clark, J.N. Smith, T.W. Speir, Cross specificity in some vertebrate and insect glutathione-transferases with methyl parathion (dimethyl p-nitrophenyl phosphorothionate), 1-chloro-2,4-dinitro-benzene and S-crotonyl-N-acetylcysteamine as substrates, Biochem. J. 135 (1973) 385–392.
[30] L.O. Nilsson, A. Gustafsson, B. Mannervik, Redesign of substrate-selectivity determining modules of glutathione transferase A1-1 installs high catalytic efficiency with toxic alkenal products of lipid peroxidation, Proc. Natl. Acad. Sci. USA 97 (2000) 9408–9412.
[31] P.L. Pettersson, A.S. Johansson, B. Mannervik, Transmutation of human glutathione transferase A2-2 with peroxidase activity into an efficient steroid isomerase, J. Biol. Chem. 277 (2002) 30019–30022.
[32] Y. Ivarsson, A.J. Mackey, M. Edalat, W.R. Pearson, B. Mannervik, Identification of residues in glutathione transferase capable of driving functional diversification in evolution. A novel approach to protein redesign, J. Biol. Chem. 278 (2003) 8733–8738.
[33] M. Scian, I. Le Trong, A.M.A. Mazari, B. Mannervik, W.M. Atkins, R.E. Stenkamp, Comparison of epsilon- and delta-class glutathione S-transferases: the crystal strructures of the glutathione S-transferases DmGSTE6 and DmGSTE7 from Drosophila melanogaster, Acta Cryst. D 71 (2015) 2089–2098.